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# Interaction plante-microorganismes : Implication de la rhizobactérie *Phyllobacterium brassicacearum* dans les réponses d'*Arabidopsis thaliana* au stress hydrique

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Justine Bresson. Interaction plante-microorganismes : Implication de la rhizobactérie *Phyllobacterium brassicacearum* dans les réponses d'*Arabidopsis thaliana* au stress hydrique. Biologie végétale. Université Montpellier II - Sciences et Techniques du Languedoc, 2013. Français. NNT : 2013MON20084 . tel-01012108

**HAL Id: tel-01012108**

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UNIVERSITE MONTPELLIER II  
Ecole Doctorale SIBAGHE – Biologie Intégrative des Plantes

THESE DE DOCTORAT  
présentée par :

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Interactions plante-microorganismes :  
Implication de la rhizobactérie  
*Phyllobacterium brassicacearum* dans les  
réponses d'*Arabidopsis thaliana*  
au stress hydrique

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Soutenue le 16 décembre 2013

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# Remerciements

*C'est quand on écrit les remerciements que l'on se dit que c'est vraiment la fin de l'aventure. Après presque 4 ans en votre compagnie, c'est avec joie mais aussi avec une pointe de tristesse que je voudrais tous vous remercier pour avoir participé à tous les niveaux à l'existence de cette thèse.*

*Avant toute chose, mes plus sincères remerciements s'adressent à Denis, mon encadrant Lepsien. Je te remercie d'avoir toujours été présent pour moi et pour la confiance que tu m'as accordée. Durant ces trois ans de thèse (et lors de mon stage de M2), tu as présenté une disponibilité sans faille pour discuter des résultats et d'interprétations, ou même de choses bien plus futiles, à n'importe quelle heure du jour ou de la nuit. Hé oui, c'est ça d'avoir un encadrant Hi-Tech ! Tu m'as toujours laissé une très grande autonomie tout en étant là pour me conseiller et me réorienter quand il le fallait. Ma thèse n'aurait jamais été ce qu'elle est aujourd'hui sans ton encadrement. J'espère avoir récupéré un peu de ta rigueur, de ton exigence et de ta passion du travail. Outre le cadre du travail, je te remercie également pour tous les agréables moments passés ensemble : les sorties, les repas et le tarot. J'espère que l'on aura encore l'occasion de travailler ensemble, mais normalement je ne compte pas te laisser tranquille avant un petit moment.*

*Je tiens également à remercier mon deuxième encadrant, Fabrice. Nos désaccords occasionnels m'ont enrichie tout autant que nos discussions scientifiques. Je tiens aussi à te remercier pour ton aide indiscutable tout au long de la thèse et durant la lourde tâche de la rédaction. Merci aussi à mon directeur de thèse, Bruno pour ton aide, ton soutien et les recadrages quand il le fallait.*

*J'ai aussi eu la chance d'avoir d'autres interlocuteurs pour me conseiller et m'épauler pendant ces trois ans. Merci Christine et Bertrand pour votre soutien et de m'avoir donné un cadre de travail idéal pour le bon déroulement de la thèse. Merci également pour nos échanges scientifiques qui ont permis de me faire évoluer dans la bonne direction.*

*J'ai passé la plus grande majorité de ma thèse au sein du LEPSE, et je vous remercie tous pour les agréables moments passés avec vous, la grande famille du LEPSE. Je ne suis pas sûre de retrouver un jour un labo aussi accueillant avec un cadre de travail exceptionnel, une chaleureuse ambiance et surtout où on peut jouer à la pétanque ou au tarot entre midi et deux.*

*Tout d'abord, je tiens à remercier l'ensemble de l'équipe SPIC. Myriam, merci pour ton aide précieuse, ta disponibilité et ton dévouement. Tu as toujours été là pour que les manip se passent au mieux et j'ai toujours pu compter sur toi au moindre problème. En plus d'être la maman de trois robots, tu es la maman des doctorants. Alex, merci pour ton aide. Le travail en ta compagnie a toujours été agréable. Merci également pour ta bonne humeur au quotidien et ton rire communicatif. Je remercie également Gaëlle pour son aide dans les manip et pour les nombreux dosages effectués. Merci à Crispulo pour les nombreuses tâches effectuées. Nous avons la chance au LEPSE ne pas se cantonner aux frontières des équipes. Je remercie ainsi l'ensemble des chercheurs avec lesquels j'ai pu échanger au cours de ces trois ans et qui m'ont tous beaucoup apporté, en particulier François T., Claude, Olivier, Christian, Angélique et Thierry.*

*Pour le côté moins scientifique mais qui rend le quotidien agréable. Merci également à Anaëlle, Maryline, Erwan, Benoit et Nathalie (Nathaliiiiie mon amour des JMJ...) pour les parties de tarot et les pauses de 15h30. Stéphane pour les brasoucades et les fêtes à Montaud. Merci à Joe, Nico et Vincent N., les geeks du labo. Joe, merci pour les canulars téléphoniques. Merci aux deux autres mamans du LEPSE, Marie-Claude et Marie-Françoise pour votre bonne humeur et votre joie de vivre communicative. Je n'oublie pas les anciens Lepsiens avec qui j'ai également passé de bons moments : Frédéric, Jessica, Christelle, Grégoire, Marie, Carine, Jorge et Mélanie. Merci aussi à Thibaut, Cloé et Marc que j'ai eu le plaisir de co-encadrer durant leur stage.*

*Je remercie également John Lunn et Regina Feil du Max Planck Institute de Golm pour leur accueil, leur gentillesse, et pour la passation du précieux protocole du dosage de tréhalose.*

*Une pensée pour mes amis BFP qui ont rendu ou qui vont bientôt rendre également leur thèse. Merci à François B, Alice et Kevin : vive le JAFK ! Merci également à Harold pour sa joie de vivre. Merci à Vincent O. pour avoir partagé la Case depuis ces trois ans, même si on ne te voit plus depuis quelques temps.*

*Puis, il y a ceux pour qui de simples remerciements ne suffiront pas pour exprimer ma gratitude. Un ENORME merci à Emilie, ma colocataire de la Case, ma confidente et amie, ma partenaire de pétage de câbles et de zumba, et mon DJ du bureau. Merci pour tous les moments que l'on a passé ensemble au labo et aussi à l'extérieur. Je n'oublierai pas nos fous rires, nos courses poursuites dans les couloirs, nos batailles dans le bureau, nos chorégraphies de 17h et j'en passe. Merci également pour ton soutien dans les derniers mois de rédaction même si je t'ai bien stressée. Je remercie également ma deuxième partenaire de zumba et amie, Aude. Merci pour nos soirées entre filles « pizza, bières », nos rigolades et d'avoir été là pour moi quand ça n'allait pas. Merci pour ton soutien et ton écoute même quand tu étais au pays des Kangourous. Vous allez beaucoup de manquer les filles ! Merci également à Cecilio et Llorenç mes deux autres lepsiens préférés. Merci Llorenç pour ton soutien, nos discussions, les organisations de soirées et de barbecue chez toi. Merci Cecilio pour les pauses thés, les parties de tarot et plus particulièrement récemment pour notre soutien mutuel pour finir nos thèses « au mieux ». J'espère que vous viendrez me voir tous les quatre !*

*Je tiens aussi à remercier mes parents qui m'ont transmis la passion des plantes et grâce à eux j'ai pu réaliser les études que je voulais. Merci à toute ma famille également. Merci à ma sœur et à ma cousine pour leur soutien téléphonique. Merci à ma grand-mère pour ses questionnements qui m'ont permis de me remettre en question : « Mais à quoi ça sert ce que tu fais ? ».*

*Enfin les derniers remerciements mais non les moindres, merci à celui que j'ai rencontré au LEPSE et qui partage maintenant ma vie. François, merci pour tout ce que l'on vit ensemble au quotidien. Merci pour ton aide indéfinissable tout au long de cette aventure, et pour nos conversations scientifiques enflammées. Merci de m'avoir toujours soutenue et encouragée pour que j'aie jusqu'au bout.*

## Résumé

Les bactéries promotrices de la croissance des plantes (PGPR) peuvent améliorer la performance et la tolérance des plantes lors de stress environnementaux. *Arabidopsis thaliana* est un modèle de choix pour étudier les mécanismes impliqués dans les interactions plante-bactéries. Nous avons analysé de multiples traits associés à la dynamique de croissance, au développement et la physiologie des végétaux afin d'évaluer les effets de l'inoculation par *Phyllobacterium brassicacearum* STM196, une PGPR isolée de la rhizosphère du colza, sur les réponses d'*A. thaliana* à des stress hydriques de différentes intensités. Grâce à des outils performants de phénotypage, nous avons développé une nouvelle approche d'analyse à haut-débit pour examiner l'implication de STM196 dans les stratégies de résistance des plantes au stress hydrique. Nos résultats montrent pour la première fois que les PGPR peuvent interférer dans les stratégies d'échappement des plantes grâce à des modifications de la croissance et du temps de floraison. De plus, STM196 induit une meilleure résistance au déficit hydrique modéré et une meilleure tolérance à la déshydratation sous une contrainte hydrique sévère. L'inoculation par STM196 peut ainsi représenter une valeur ajoutée aux stratégies de résistance intrinsèques aux plantes, ce qui est illustrée par sa remarquable capacité à promouvoir la survie et la production de biomasse végétale dans des environnements contrastés. Nos résultats soulignent l'importance des interactions plantes-bactéries dans les réponses des plantes à la sécheresse et offrent de nouvelles voies de recherches pour l'amélioration de la résistance à la sécheresse dans les cultures.

**Mots clés :** Bactéries promotrices de la croissance des plantes (PGPR), *Arabidopsis thaliana*, *Phyllobacterium brassicacearum* (STM196), interaction plante-bactérie, déficit hydrique, stratégies de résistance des plantes.

## Abstract

Plant growth promoting rhizobacteria (PGPR) can enhance plant performance and plant tolerance to environmental stresses. *Arabidopsis thaliana* is a useful organism to study the mechanisms involved in plant-PGPR interactions. We analyzed multiple plant traits related to growth dynamics, development and physiology in order to assess the effects of *Phyllobacterium brassicacearum* STM196 strain, isolated from the rhizosphere of oilseed rape, on *Arabidopsis* responses to well-defined soil water availability. Using powerful tools for phenotyping, we developed a new high-throughput analysis to examine the implication of STM196 on plant strategies to cope with water stress. Our results show for the first time that PGPR can interfere in escape strategies of plants through modifications in plant growth and flowering time. Moreover, STM196 induced a better resistance to moderate water deficit and a better tolerance to dehydration under a severe stress. Inoculation by STM196 can represent an added value to plant resistance strategies, as illustrated by its remarkable ability to promote plant survival and biomass production under contrasted environments. Our results highlight the importance of plant-bacteria interactions in plant responses to drought and provide a new avenue of investigations to improve drought resistance in crops.

**Key words:** Plant growth promoting rhizobacteria (PGPR), *Arabidopsis thaliana*, *Phyllobacterium brassicacearum* (STM196), plant-bacteria interactions, water deficit, plant resistance strategies.





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Vile D, Pervent M, Belluau M, Vasseur F, Bresson J, Muller B, Granier C, Simonneau T. 2012. Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? Plant Cell and Environment 35: 702-718.



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# ***Introduction générale***

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# *Introduction générale*

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Selon le Programme Alimentaire Mondial, il est indispensable dans les années à venir d'améliorer la production agricole de 70 à 100% pour nourrir la population globale qui ne cesse d'augmenter et devrait atteindre 9 milliards d'individus en 2050. Les longues périodes de sécheresse, qui sont à la hausse ces dernières années, sont considérées comme les premières causes naturelles responsables de problèmes majeurs dans les cultures. En effet, dans un contexte de changements climatiques, le réchauffement actuel a un impact conséquent sur la disponibilité en eau dans de nombreuses régions. Les écosystèmes subissent ainsi des carences hydriques notamment dans les pays du Sud. Selon le rapport du Groupe d'experts intergouvernemental sur l'évolution du climat (GIEC, 2007), on peut déjà noter à l'échelle du globe une hausse des températures moyennes de l'atmosphère touchant une multitude de systèmes naturels. Les estimations les plus probables du réchauffement sont comprises entre 1,8 °C et 4 °C d'ici 2100.

Hors contexte des changements globaux, l'agriculture est souvent pratiquée dans des régions où la disponibilité en eau est fréquemment déficiente comparée à l'évapotranspiration, limitant la croissance et la productivité des plantes (Lawlor, 2012). Le déficit hydrique à long terme est un problème majeur mais aussi à court terme où il induit une diminution de la production des plantes même si les conditions redeviennent favorables. L'importance sociale et économique d'une meilleure production des plantes lors d'un déficit hydrique est énorme, et dans un tel contexte, il est important de comprendre les stratégies déployées par les plantes pour faire face à la sécheresse. En conditions naturelles, de nombreux paramètres peuvent influencer les capacités d'acclimatation des plantes face à des stress abiotiques. Parmi ceux-ci peuvent être cités la qualité physico-chimique du sol ou la présence de micro-organismes pouvant agir sur la disponibilité des ressources ou sur le fonctionnement des plantes. Souvent les bactéries sont perçues comme des germes responsables de maladies. En réalité, très peu des milliers de sortes de bactéries peuplant la Terre sont pathogènes. La plupart d'entre elles sont bénéfiques, voire essentielles au maintien de la vie. Les bactéries sont omniprésentes et peuvent vivre dans des milieux très variés aux conditions environnementales pouvant être



extrêmes, en association ou non avec d'autres organismes. Certaines bactéries présentes dans le sol, dites rhizobactéries, sont capables d'interagir avec système racinaire des plantes. Les interactions bénéfiques entre plantes et bactéries peuvent prendre différentes formes et améliorer la production des plantes. Les bactéries fixatrices d'azote qui vivent en symbiose avec de nombreuses plantes de la famille des Légumineuses, présentent un intérêt agronomique et environnemental. On estime que la moitié de la fixation biologique d'azote est réalisée par cette symbiose, avec un taux allant de 10 à 300 kg d'azote fixé par hectare et par an (Lindstrom et al., 2010). D'autres bactéries rhizosphériques, regroupées sous le terme "Plant Growth-Promoting Rhizobacteria" (PGPR), suscitent un intérêt croissant. Ces bactéries en interaction mutualiste avec les plantes n'induisent pas la formation d'un organe spécifique et présentent l'avantage d'avoir une spécificité d'hôte moindre. Ces bactéries sont capables, comme leur nom l'indique, de stimuler la croissance des plantes mais elles permettent aussi aux plantes de résister à des stress biotiques et/ou abiotiques. Différentes formulations à base de PGPR sont de nos jours commercialisées et utilisées en agriculture (Lucy et al., 2004; Babalola, 2010). Ces interactions peuvent ainsi représenter un enjeu majeur pour l'amélioration de la performance des plantes en condition de sécheresse, et une stratégie d'avenir pour optimiser les cultures.

Au cours de ces dernières années, de nombreuses études sur les effets des PGPR sur la croissance et la résistance des plantes face à des stress biotiques et abiotiques ont été largement menées (pour revues, van Loon, 2007; Lugtenberg & Kamilova, 2009). L'utilisation d'*Arabidopsis* comme plante modèle dans l'analyse des interactions plante-rhizobactéries a permis une avancée dans la compréhension des mécanismes impliqués, notamment dans les signalisations hormonales et les réseaux de gènes impliqués (pour revue, Desbrosses *et al.*, 2012). Cependant, peu d'études se sont intéressées à la compréhension de l'effet des PGPR sur le développement et la physiologie des plantes lors d'un stress hydrique.

Les recherches que j'ai menées durant ma thèse s'intéressent à la résistance des plantes au déficit hydrique via l'interaction plante-microorganismes. Le couple entre la plante modèle *Arabidopsis thaliana* et la souche *Phyllobacterium brassicaceraum* STM196 a été utilisé. La souche STM196, isolée de la rhizosphère du colza, permet une stimulation de la croissance chez *A.thaliana* et engendre des réponses typiques des PGPR (modifications de l'architecture racinaire, de la signalisation hormonale et implication dans la nutrition azotée) en condition optimale de croissance *in vitro* (Contesto *et al.*, 2008; Contesto *et al.*, 2010; Galland *et al.*, 2012; Kechid *et al.*, 2013). Ici, nous avons utilisé la plateforme de phénotypage PHENOPSIS

(Granier *et al.*, 2006) afin d'analyser l'implication de la souche STM196 sur la dynamique de la croissance et la physiologie d'*Arabidopsis* en conditions édaphiques stressantes. Un stress hydrique modéré et un stress sévère ont été appliqués aux plantes afin de comprendre l'effet de STM196 sur les différentes stratégies mises en place par les plantes lors d'un stress hydrique du sol.

La synthèse bibliographique présentée dans le **Chapitre 1** donne l'état de nos connaissances actuelles sur les mécanismes de résistances des plantes face au déficit hydrique, et l'implication des rhizobactéries dans ces mécanismes. Lors d'un stress hydrique modéré, les plantes ont la capacité de maintenir leur statut hydrique via des mécanismes maximisant le prélèvement de l'eau dans le sol et réduisant les pertes d'eau. Lors d'un stress hydrique sévère, les plantes doivent mettre en place des mécanismes de protection contre la déshydratation des tissus pour éviter leur sénescence pouvant entraîner la mort des plantes. L'inoculation par des rhizobactéries peut permettre une amélioration de la résistance des plantes via leurs actions dans les stratégies intrinsèques aux plantes. La stimulation de la croissance chez *A.thaliana* par STM196 a été montrée au sein de l'équipe dirigée par Bruno Touraine, et divers mécanismes ont pu être mis en évidence illustrant la multiplicité des réponses occasionnées chez les plantes lors de l'inoculation par une PGPR.

Le **Chapitre 2** présente le matériel et les méthodes générales utilisés tout au long de cette étude. Cette partie est suivie par un article d'opinion publié dans la revue WIREs Developmental Biology (2013). Cet article propose des recommandations et présente les pièges à éviter dans le phénotypage à haut débit du développement foliaire. De plus, il présente l'importance d'une analyse dynamique de la croissance à haut débit dans les interactions plante-rhizobactéries. En effet, de nombreuses études s'intéressent à l'effet de l'inoculation par des rhizobactéries sur la croissance des plantes à un point donné dans le temps, pouvant correspondre à une date donnée après inoculation et/ou germination. Cependant, dans notre cas particulier nous avons pu montrer qu'étudier l'effet promoteur de STM196 sur la croissance d'*A. thaliana*, sans prendre en compte une analyse temporelle peut conduire à des conclusions biaisées.

Le **Chapitre 3** présente l'implication de la souche STM196 dans les réponses d'*A. thaliana* lors d'un stress hydrique modéré (article scientifique publié dans New Phytologist, 2013). Nous avons montré que l'inoculation par STM196 induit une amélioration de la production de biomasse chez *A. thaliana* lors d'un déficit hydrique et ainsi une meilleure résistance des plantes. L'inoculation par STM196 entraîne des modifications physiologiques

et développementales des plantes. STM196 interfère dans les mécanismes de résistance d'Arabidopsis au déficit hydrique, notamment par des modifications dans la dynamique de croissance, le taux de transpiration et la production d'ABA, permettant à terme une meilleure utilisation de l'eau chez les plantes inoculées. De plus, cette étude a mis en évidence pour la première fois un retard de floraison chez les plantes inoculées participant à la mise en place d'une meilleure résistance.

Par la suite, nous nous sommes intéressés à l'effet de la souche STM196 sur la survie d'Arabidopsis lors d'un stress hydrique sévère amenant jusqu'à la mortalité des plantes (**Chapitre 4** sous forme d'un manuscrit en préparation). Différents scénarios de stress ont été utilisés avec un dessèchement du sol contrôlé jusqu'à une humidité donnée suivie d'une ré-irrigation jusqu'à atteindre une humidité optimale pour la croissance. Sur cinq stress hydriques sévères imposés aux plantes, trois ont induit une forte mortalité des plantes allant de 60 à 80% chez les plantes non inoculées. L'inoculation par la souche STM196 a induit une augmentation significative de la survie des plantes dans tous les scénarios affectant la survie des plantes. Au cours de cette étude, nous avons mesuré la fluorescence de la chlorophylle et utilisé la mesure de l'efficacité de l'appareil photosynthétique II ( $F_v/F_m$ ) comme outil d'analyse de l'état physiologique des plantes. Cette étude a permis de mettre en évidence une meilleure tolérance à des dommages photosynthétiques chez les plantes inoculées, pouvant être permis par un retard de la déshydratation des tissus au cours du dessèchement du sol et aussi une meilleure tolérance à des faibles teneurs en eau dans les feuilles chez les plantes inoculées.

Des études préliminaires au sein de l'équipe de Bruno Touraine ont mis en évidence l'implication de STM196 dans le métabolisme du tréhalose. De plus, le tréhalose, étant un sucre non réducteur largement impliqué dans le développement et les réponses des plantes au déficit hydrique (pour revue, Paul *et al.*, 2008), nous avons émis l'hypothèse du tréhalose comme molécule clé dans les réponses occasionnées chez *A. thaliana* lors de l'inoculation par STM196 (**Chapitre 5**). Nous avons utilisé une approche mutant/sauvage pour le seul gène connu de dégradation du tréhalose, la tréhalase afin d'appréhender l'implication du tréhalose dans les réponses induites par la bactérie.

Enfin, nous avons voulu explorer la diversité des effets bactériens sur les réponses d'Arabidopsis au stress hydrique (**Annexe 1** sous forme de rapport de stage). Pour cela, nous avons utilisé différentes souches bactériennes de la classe des Protéobactéries dont leur interaction avec *A. thaliana* n'est pas connue.

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# ***Chapitre 1***

## ***Synthèse bibliographique***

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# ***Synthèse bibliographique***

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## ***I. La résistance au stress hydrique, un enjeu majeur pour l'agriculture***

### **I.1. Mécanismes mis en place par les plantes pour faire face au déficit hydrique**

La résistance au déficit hydrique peut être permise par différentes stratégies qui ne sont pas strictement indépendantes, mais qui peuvent être définies par des traits quantitatifs distincts (Blum, 2005, 2011). En condition de sécheresse, les plantes subissent diverses modifications phénologiques, morpho-physiologiques et métaboliques. Généralement, trois aspects peuvent être considérés : l'échappement, l'évitement de la déshydratation, la tolérance à la déshydratation et la survie selon la sévérité du stress (Fig. 1, Farooq *et al.*, 2009; Verslues & Juenger, 2011; Lawlor, 2012).

#### ***I.1.1. Le mécanisme d'échappement est lié à des modifications de la phénologie des plantes***

Le cycle de vie des plantes peut être considéré comme l'intégration de plusieurs phases de développement délimitées par des stades distincts tels que la germination, l'émergence successive des diverses feuilles, l'initiation de la floraison, la floraison et la fructification. La phénologie des plantes rend compte de l'intégration des vitesses de développement au cours du temps jusqu'à un point final spécifique qui correspond souvent au début de la floraison (McMaster *et al.*, 2009). Chez *Arabidopsis*, il existe une nomenclature de la phénologie permettant une description précise du stade de développement (Boyes *et al.*, 2001). La phénologie des plantes est affectée en réponse au déficit hydrique et ces réponses peuvent être

Sévérité du déficit hydrique

### Echappement

Réponses physiologiques: ajustement du développement (très grande plasticité) et maximisation de la croissance avant les effets négatifs du stress hydrique

Réponses métaboliques: investissement minimale dans les voies de protection au stress et activité maximale des voies impliquées dans la croissance

Traits majeurs: temps de floraison, vitesse de croissance plante entière et à l'échelle de la feuille, vitesse de germination, réallocation des ressources vers les fruits

Implications des PGPR: très peu connu de nos jours. Accélération de la vitesse de croissance, et raccourcissement de la période végétative, floraison précoce

### Evitement de la déshydratation

Réponses physiologiques: ajustement de traits permettant de limiter les pertes en eau et maintenir un statut hydrique compatible à un bon fonctionnement métabolique.

Réponses métaboliques: investissement dans les voies de protection au stress avec une activité métabolique forte

Traits majeurs: ralentissement de la croissance, transpiration par unité de surface (fermeture stomatique), morphologie des feuilles et de la racine, ratio racine-feuille, ajustement osmotique

Implications des PGPR: maximisation des réponses des plantes : appareil racinaire plus efficace, amélioration des réduction des pertes en eau (via modifications dans le métabolisme de l'ABA), augmentation des capacités photosynthétiques. Diminution de la sensibilité des plantes via réduction éthylène *in planta*

### Tolérance à la déshydratation/Survie

Réponses physiologiques: ajustement de traits permettant de survivre à la dessiccation des tissus

Réponses métaboliques: investissement dans les voies de protection des tissus avec une croissance diminuée, voire arrêtée (cas des plantes de résurrection) Coût métabolique très fort.

Traits majeurs: accumulation de métabolites et de protéines protecteurs, osmo-protection, stabilité des membranes, forte diminution/arrêt de la croissance, sénescence

Implications des PGPR: activation des mécanismes d'osmo-protection amélioration de la survie (via accumulation de tréhalose)

**Fig. 1** Différentes stratégies d'adaptation à la sécheresse allant de l'échappement à la survie des plantes selon la sévérité du stress hydrique et les implications des PGPR dans ces stratégies (selon Verslues & Juenger, 2011).

considérées comme des mécanismes d'échappement au stress. L'échappement à la sécheresse est observé dans le cas où les plantes ajustent leur développement au cours du temps afin d'avoir un cycle de vie complet en évitant ou diminuant l'impact du stress (Verslues & Juenger, 2011; Assmann, 2013). Autrement dit, l'échappement est caractérisé par le succès de la reproduction avant l'apparition des effets négatifs du stress. Le majeur trait impliqué dans la stratégie d'échappement est le temps de floraison (Passioura, 1996; Araus *et al.*, 2002). De plus, ce trait, fortement relié à des adaptations à la sécheresse, est un évènement clé qui détermine la production de biomasse et donc le rendement des plantes (Jung & Muller, 2009).

En réponse au stress hydrique, la floraison des plantes peut être retardée (McMaster *et al.*, 2009; Tisne *et al.*, 2010) ou avancée (Verslues & Juenger, 2011) en fonction de l'espèce et du génotype considérés et de l'occurrence, de la durée et de la sévérité du stress (McMaster *et al.*, 2009). Chez les céréales, la floraison est sensible aux conditions hydriques du sol (Winkel *et al.*, 1997). Par exemples, chez le blé et l'orge, l'anthèse (période pendant laquelle la fleur est complètement ouverte et fonctionnelle) et la maturation du fruit ont lieu 13 et 15 jours plus tôt en condition de stress hydrique sévère (McMaster & Wilhelm, 2003). A l'inverse, chez le maïs, l'anthèse et l'apparition des soies (appareil reproducteur femelle) ont lieu un peu plus tard en réponse au déficit hydrique (Campos *et al.*, 2004). Chez *Arabidopsis*, cultivées en laboratoire, il est communément observé que les contraintes, y compris la sécheresse, conduisent à une floraison plus rapide (Assmann, 2013).

Au cours de ces dernières années, de nombreuses études génétiques des plantes ont été menées afin d'appréhender les voies impliquées dans les mécanismes de floraison (pour revues Koornneef *et al.*, 1998; Bernier & Perilleux, 2005; Jung & Muller, 2009). L'altération des mécanismes endogènes impliqués dans la floraison est une des stratégies pour augmenter le rendement des plantes cultivées. Néanmoins, il est important de noter que la floraison n'est pas un évènement indépendant et est relié à la croissance. Plusieurs études rapportent le fait que la vitesse de croissance et la durée des différentes phases du développement sont dépendantes du temps de floraison (Steynen *et al.*, 2001; Salehi *et al.*, 2005). Des étroites relations entre la production des feuilles, la croissance individuelle des feuilles et le temps de floraison ont aussi été mises en évidence (Cookson *et al.*, 2007; Tisne *et al.*, 2008).

De plus, le succès de l'échappement au déficit hydrique repose sur une meilleure reproduction mais également par une allocation efficace des ressources vers les fruits et les graines. Ceci est associé à la capacité des plantes à stocker des réserves dans des organes spécialisés et les mobiliser pour la production de fruits (Bruce *et al.*, 2002). La réallocation des ressources vers les parties fructifères est augmentée lors du déficit hydrique (Yang *et al.*,



2001). En cas de stress hydrique prolongé, leur potentiel biologique peut être conservé sous la forme d'un organe (graine, bulbe, rhizome) qui garde la capacité de régénérer de nouveaux individus lors de conditions favorables. Enfin, les plantes sont susceptibles de mettre en place des mécanismes d'échappement de manière très précoce. En effet, des modifications de la vitesse de germination ont été également considérées comme de l'échappement (Clerkx *et al.*, 2004; Verslues & Juenger, 2011).

Les plantes présentant essentiellement des mécanismes d'échappement possèdent une très forte plasticité du développement mais semblent être mal équipées pour tolérer la déshydratation réelle des tissus (Chaves *et al.*, 2003).

### *1.1.2. L'évitement de la déshydratation*

L'évitement de la déshydratation est observé chez les plantes qui grandissent en période de stress mais qui maintiennent un statut hydrique tissulaire compatible avec un fonctionnement métabolique, retardant et/ou minimisant les effets négatifs induits par le manque d'eau. La déshydratation des cellules des tissus foliaires et racinaires, se traduit communément par une cavitation exacerbée du xylème entraînant la mort cellulaire (Schulze, 1986; Chaves, 1991). L'évitement à la déshydratation est une stratégie commune aux plantes annuelles et pérennes et est associé à différents traits adaptatifs (Chaves *et al.*, 2003). Différents mécanismes peuvent être mis en place par la plante afin d'une part, de réduire les pertes en eau des tissus et de protéger les cellules de la déshydratation et d'autre part de maximiser le prélèvement de l'eau du sol.

#### *Réduction des pertes d'eau*

Un des traits majeurs impliqués dans les réponses au déficit hydrique est la conductance stomatique permettant de réduire les pertes en eau des tissus et de maintenir un potentiel hydrique foliaire le plus haut possible. La conductance stomatique est reliée au mouvement stomatique : une diminution de la conductance traduit une fermeture stomatique, et à l'inverse une augmentation de la conductance traduit une ouverture stomatique. Les stomates permettent une régulation fine des échanges de vapeur d'eau et de CO<sub>2</sub> contrôlant ainsi le taux de transpiration et de photosynthèse (Farquhar & Sharkey, 1982). De façon générale, la conductance stomatique et l'assimilation carbonée sont étroitement liées. Des modifications de la transpiration peuvent induire des changements de la température et du potentiel hydrique des feuilles. La diminution de la transpiration peut participer à l'augmentation de la

température des tissus. L'échauffement des tissus foliaires affecte alors rapidement la photosynthèse instantanée (Bernacchi *et al.*, 2001).

L'acide abscissique (ABA) a été identifié comme un des signaux chimiques impliqués dans la régulation stomatique (Schulze, 1986; Christmann *et al.*, 2007; Tardieu *et al.*, 2010). Il a été montré que la production endogène, un apport exogène dans le sol ou injecté dans la tige conduit à la fermeture stomatique (pour revue voir Davies & Zhang, 1991). L'ABA joue le rôle de signal chimique entre les racines et les feuilles (Davies & Zhang, 1991), et interagit aussi avec d'autres molécules signaux de communication entre organes tel que l'éthylène (Wilkinson & Davies, 2002). Cependant, la régulation stomatique via l'ABA n'est pas simple et implique des transports à longue distance mais également des modulations de la concentration en ABA des cellules de gardes (Wilkinson & Davies, 2002).

D'autres hormones peuvent être impliquées dans la régulation stomatique soit de manière indépendante soit en action avec ABA. Par exemple, l'augmentation de la concentration en cytokinine dans le xylème chez le coton diminue la sensibilité des stomates à l'ABA, et ainsi induit l'ouverture stomatique (Radin *et al.*, 1982).

La conductance stomatique représente un trait majeur au niveau de la feuille pour réduire les pertes en eau. Cependant, les changements morphologiques de la feuille ne sont pas négligeables (O'Toole J & Cruz, 1980). La minimisation des pertes d'eau peut également être produite par la réduction de l'absorption de la lumière via l'enroulement des feuilles notamment chez le maïs et le riz (Fernandez & Castrillo, 1999; Kondo *et al.*, 2000). De même, la densification des trichomes (Larcher, 2000), ou des changements dans l'angle des feuilles (Vasseur *et al.*, 2011) permettent d'augmenter la réflectance. D'autres modifications au niveau des surfaces d'échange peuvent intervenir notamment une baisse de la densité stomatique, ou l'épaississement de la cuticule imperméabilisant les surfaces (Shepherd & Wynne Griffiths, 2006) et altérant les propriétés d'échange pour réduire le "budget" en eau.

Au niveau de la croissance, le ralentissement de l'expansion foliaire limite en soi la surface d'échange. Réduire la taille des plantes et la surface foliaire permet de réduire la consommation et la perte d'eau par les feuilles (Tardieu *et al.*, 2011). Récemment, nous avons montré que la taille de différents écotypes d'*Arabidopsis* est corrélée négativement à résistance à la sécheresse (Vile *et al.*, 2012; *Annexe 2*).

### *Prélèvement de l'eau dans le sol*

Le prélèvement de l'eau du sol peut être maximisé par un ajustement des allocations des ressources vers les parties racinaires (Jackson *et al.*, 2000). En effet, une augmentation du

ratio racine : feuille est souvent observée, via une réduction de la croissance des parties aériennes et/ou par une augmentation de la croissance racinaire (Boyer, 1985). De plus, les caractéristiques morphologiques du système racinaire telles que la biomasse, la longueur, la densité ou la profondeur sont des traits qui contribuent également à la production lors d'un stress hydrique (Turner *et al.*, 2001; Farooq *et al.*, 2009). L'augmentation de ces différents traits permet un maintien du prélèvement de l'eau, mais aussi une exploration du sol plus étendue pour trouver de nouvelles sources d'eau. La réduction des pertes d'eau et l'augmentation de la capacité d'absorption depuis le sol contribue tous deux à une meilleure efficacité de l'utilisation de l'eau.

### *L'ajustement osmotique*

L'accumulation d'osmo-protecteurs est très importante pour faire face aux dommages occasionnés par le stress et pour maintenir une teneur en eau compatible au bon fonctionnement des cellules (Djilianov *et al.*, 2005). Ces solutés sont des composés à faible poids moléculaire, très solubles et ne sont pas toxiques pour les cellules même à de très fortes concentrations. Généralement, ils protègent les plantes par leur participation dans les ajustements osmotiques, la détoxification des espèces réactives de l'oxygène et la stabilisation des membranes (Farooq *et al.*, 2009).

L'ajustement osmotique est un trait important pour maintenir le statut hydrique des plantes (Taiz, 2006) et pour retarder la déshydratation des tissus lors d'un stress hydrique (Morgan, 1990). L'ajustement osmotique est principalement due à l'accumulation d'osmolytes de différentes natures tels que des sucres (e.g. trehalose, mannitol, saccharose, fructane), la proline, la glycine-bétaïne, des acides organiques, le calcium, le potassium ou encore certains ions. Lors d'un déficit hydrique, l'accumulation de ces solutés confère un potentiel osmotique plus faible aux cellules, attirant ainsi l'eau à l'intérieur des cellules et permettant alors de conserver la turgescence cellulaire (Gibon *et al.*, 1997). Une turgescence cellulaire à des niveaux plus élevés contribue à l'amélioration des performances des plantes et au maintien des processus physiologiques tels que l'ouverture stomatique, la photosynthèse et l'expansion foliaire (Subbarao *et al.*, 2000; Serraj & Sinclair, 2002). De plus, l'accumulation d'osmolytes a été utilisée comme un critère de sélection dans des programmes d'amélioration génétiques des cultures pour améliorer le rendement du grain de riz dans des environnements défavorables (Nguyen *et al.*, 1997).

### I.1.3. La tolérance à la déshydratation et la survie des plantes lors d'un stress hydrique sévère

Dans le cas d'un stress hydrique modéré ou d'un stress hydrique à courte durée, les mécanismes d'évitement peut être suffisants pour maintenir la teneur en eau des plantes, mais si le stress persiste ou devient plus sévère les plantes ne sont plus capables de maintenir leur statut hydrique et doivent alors tolérer des potentiels hydriques plus faibles. Les mécanismes sous-jacents à la tolérance à la déshydratation sont étroitement liés aux mécanismes d'évitement et les processus sont souvent interchangeable (Lawlor, 2012). Cependant, les plantes doivent faire face à des dommages cellulaires plus importants affectant l'intégrité cellulaire. Dans les cas les plus extrêmes, un fort déficit hydrique peut conduire à la mortalité des plantes.

#### *Ralentissement/arrêt de la croissance*

Le cas le plus spectaculaire de survie à des fortes déshydratations des tissus concerne les plantes de résurrection (Moore *et al.*, 2009). Ces plantes peuvent tolérer une déshydratation presque complète de leur tissus, on parle alors de tolérance à la dessiccation. Ces plantes accumulent à de très fortes concentrations des protéines protectrices, des solutés permettant la stabilisation des membranes et des antioxydants (Moore *et al.*, 2009). Le coût métabolique pour maintenir un tel équilibre métabolique est élevé et conduit à une vitesse de croissance très faible voire arrêtée (Verslues *et al.*, 2006). En effet, il a été montré chez *Arabidopsis* que la croissance est fortement réduite au cours du dessèchement du sol (Lechner *et al.*, 2008). L'arrêt de croissance est relié à des modifications de l'extensibilité de la paroi cellulaire qui devient plus rigide (Peleman *et al.*, 1989; Ingram & Bartels, 1996). Cependant, lorsque les conditions redeviennent favorables les cellules épidermiques des feuilles retrouvent leur habilité à croître (Lechner *et al.*, 2008). Les plantes peuvent aussi présenter des changements morphologiques des feuilles. Des feuilles plus petites, épaisses et avec une faible surface spécifique des feuilles contribuent à une meilleure résistance à des conditions défavorables (Maroco *et al.*, 2000).

#### *L'osmo-protection des tissus et la stabilisation des membranes*

Lors d'un déficit hydrique, les plantes doivent faire face à des détériorations tissulaires, notamment par l'accumulation d'espèces réactives de l'oxygène (ROS) telles que par exemple des radicaux libres, des ions oxygénés et des peroxydes. Les ROS interagissent avec les

protéines, les lipides et l'ADN, induisant ainsi des dommages oxydatifs et compromettant le fonctionnement normal des cellules. Les dommages oxydatifs sont diminués par l'action combinée d'osmo-protecteurs tels que la cystéine, le glutathion et l'acide ascorbique, mais aussi par l'activité de diverses enzymes (superoxyde dismutases, catalases, peroxidases, ascorbate peroxidases et la glutathion reductase; Hasegawa *et al.*, 2000; Prochazkova *et al.*, 2001).

Les osmo-protecteurs ont aussi un rôle important dans la stabilité des membranes, qui est fortement affectée lors de stress abiotiques. Les membranes sont les premières cibles des processus de dégradation et il a été montré que le contenu lipidique diminue progressivement lors d'un stress (De Paula *et al.*, 1990). La stabilité de la membrane des cellules est largement utilisée comme un index physiologique de l'état des plantes lors d'un stress hydrique (Bajji *et al.*, 2002). Il a été montré chez *Arabidopsis* que le maintien de la teneur en lipides et la stabilité de leur composition lui confère une meilleure résistance des membranes face au stress hydrique (Gigon *et al.*, 2004). Un large éventail de solutés ont été identifiés pour leur capacité à limiter les dommages causés aux membranes, tels que la proline, le glutamate, la glycine bêtaïne ou encore le mannitol et le tréhalose (Hoekstra *et al.*, 2001).

### *La senescence*

Lorsque le stress hydrique devient trop sévère les tissus se nécrosent jusqu'à la mort possible de l'individu malgré un retour à des conditions favorables. Cette nécrose peut être expliquée par une forte diminution des capacités photosynthétiques et donc à une carence en carbone via la fermeture stomatique (McDowell, 2011). En effet, lors d'un stress sévère, les plantes doivent faire face à un compromis entre réduire les pertes en eau et optimiser leur alimentation carbonée via la régulation stomatique. La senescence des feuilles âgées contribue ainsi à économiser de l'eau, et à pallier à une carence en carbone par un programme de recyclage dans la plante permettant une réallocation des nutriments stockés dans les vieilles feuilles vers les jeunes feuilles. Les vieilles feuilles subissant le stress deviennent donc une source de carbone pour les jeunes feuilles (Chaves *et al.*, 2003). De plus, la senescence des feuilles peut permettre de réduire la surface d'échange et de limiter la perte d'eau.

Les plantes présentent donc de nombreuses réponses aux déficits hydriques (Fig. 1). Cette multiplicité des réponses peut être expliquée par les différentes stratégies employées par les plantes pour survivre à la sécheresse. Toutefois, tous ces mécanismes de résistance ne sont pas mutuellement exclusifs et peuvent être présents chez une même plante agissant en synergie (Chaves *et al.*, 2003). L'amélioration de la résistance des plantes passent donc par la compréhension de ces mécanismes et les traits qui leur sont associés. Cependant, beaucoup de traits associés à la résistance à la sécheresse présentent un double effet, pouvant être positifs lors d'un scénario sévère de dessèchement des sols et négatifs lors d'un stress plus modéré, ou vice versa (Tardieu, 2012). Il est donc difficile de choisir un trait en particulier pour l'amélioration de la résistance des plantes. Actuellement, l'inoculation du sol par des rhizobactéries présentent un intérêt croissant (Lucy *et al.*, 2004) dans l'agriculture et permettent une approche plus intégrative de par leur action sur le fonctionnement global des plantes.

## II. Les Plant Growth Promoting Rhizobacteria (PGPR), une interaction mutualiste avec les plantes

Beijerinck (1888) a été le premier à isoler une bactérie à partir des nodules racinaires de plantes. A la fin du 19<sup>e</sup> siècle, Franck (1889) a nommé cette bactérie *Rhizobium leguminosarum* signifiant "qui vit dans les racines" et a identifié également diverses espèces appartenant à ce même groupe. La famille des *Rhizobiaceae* est constituée par un ensemble hétérogène de bactéries aérobies en bâtonnet gram-négatif. Ces bactéries ont la capacité d'interagir avec les plantes, dont certaines peuvent infecter les racines, et parfois les tiges des légumineuses pour former des nodules. De Bary (1979) a défini cette interaction de *symbiose* comme la vie en commun de deux espèces différentes, la plus grande étant appelée l'hôte et la plus petite le symbionte. La notion de symbiose est de nos jours communément utilisée pour un type d'interaction liant étroitement la plante hôte et son symbionte, et amenant à la formation d'un organe spécifique, le nodule.

Jusqu'à récemment ces rhizobactéries ont été exclusivement regroupées au sein de la sous-classe des *Alphaproteobacteria* dans l'ordre de *Rhizobiales* qui inclue aussi des espèces qui ne sont pas des symbiontes des légumes (Laranjo *et al.*, 2013). Cependant, l'identification de bactéries fixatrices d'azote symbiontes d'espèce sauvages de légumineuses dans la sous-classe des *Betaproteobacteria* a révélé la grande diversité taxonomique des *rhizobia* (Moulin *et al.*, 2001). De plus, un groupe particulier de bactéries, appartenant principalement aux

*Alphaprotéobacteria* et n'induisant pas la formation de nodule chez les plantes a été mise en évidence (Kloepper & Schroth, 1978). Ces bactéries par leur capacité à stimuler la croissance des plantes ont été ainsi nommées Plant Growth Promoting Rhizobacteria (PGPR). Leur interaction avec les plantes n'est pas définie comme une symbiose, au sens véritable du terme, mais plutôt comme une interaction mutualiste, dans laquelle les deux organismes impliqués tirent profit de cette relation non obligatoire. Les PGPR ont la particularité d'être présentes à la fois à l'intérieur des tissus végétaux, dites endophytiques, mais peuvent également se situer à la surface des racines (rhizoplan). De plus, un même genre bactérien peut infecter différemment selon l'espèce végétale présente. Il a été montré que la bactérie *Azospirillum spp.* est généralement située dans la rhizosphère des racines de blé, mais elle est parfois trouvée à l'intérieur des racines (Rothballer *et al.*, 2003).

Lors d'une interaction mutualiste, les bactéries bénéficient d'un apport en nutriments via les exsudats racinaires secrétés par les plantes (Lugtenberg & Kamilova, 2009). Il a été montré que 5 à 21% du carbone fixé par les plantes est secrété principalement par les exsudats racinaires dans la rhizosphère (Clarkson, 1996). De plus, la nature de ces exsudats peut participer à l'attraction et à la sélection des bactéries présentes dans la rhizosphère (Micallef *et al.*, 2009). Les bactéries utilisent donc ces ressources carbonées mais secrètent elles-mêmes des métabolites dans la rhizosphère, qui peuvent être perçus par les cellules racinaires de la plante hôte et jouer le rôle de molécules signal (Bais *et al.*, 2004). Le mécanisme de reconnaissance et de communication entre les rhizobactéries symbiotiques et les légumineuses est largement décrit de nos jours (Desbrosses & Stougaard, 2011), par contre celui entre les PGPR et leur plantes hôtes reste encore méconnu. Ceci peut être expliqué par la capacité des PGPR à interagir avec une large variété de plantes. Il a été reporté que les genres *Azotobacter*, *Pseudomonas* et *Bacillus* peuvent promouvoir la croissance des plantes telles que le blé (Abbass & Okon, 1993), le maïs (Berge *et al.*, 1990) et le colza (Hong *et al.*, 1991). De plus, le genre *Azospirillum* est largement connu pour interagir avec de nombreuses plantes (Bashan *et al.*, 2004)

Les PGPR participent par des modifications globales du fonctionnement des plantes à l'amélioration de la santé des plantes. Plus particulièrement, certaines PGPR induisent une amélioration des résistances des plantes à des stress abiotiques et biotiques. Ces bactéries sont capables d'interférer dans les réponses des plantes aux contraintes environnementales de façon direct ou indirectement, et de leur conférer de nouvelles capacités. Au cours de cette étude, nous nous sommes intéressés aux implications des PGPR dans les réponses des plantes soumises à un déficit hydrique.

## II.1. Les PGPR interfèrent dans les mécanismes de réponses des plantes lors d'un stress hydrique

L'implication des PGPR dans la tolérance des plantes lors d'un déficit hydrique a largement été étudiée (pour revues, Dimkpa *et al.*, 2009; Yang *et al.*, 2009). Ici, nous présentons comment les PGPR peuvent interagir dans les stratégies des plantes pour faire face à un déficit hydrique (Fig. 1).

### II.1.1. Les PGPR peuvent moduler la phénologie des plantes

Indubitablement, les PGPR stimulent la croissance des plantes. Cependant, malgré la pléthore d'études existantes sur le sujet, très peu d'entre elles se sont intéressées à l'analyse dynamique de l'effet des PGPR sur la croissance des plantes. L'effet promoteur de croissance des plantes a souvent été démontré à une date donnée après semis ou après inoculation. Communément, il a été montré que les PGPR permettent une accélération du développement précoce des plantes, amenant à terme à une production plus importante des plantes (Ryu *et al.*, 2003; Jaleel *et al.*, 2007; Zahir *et al.*, 2008). Ces études ont été récemment complétées par la mise en évidence de l'implication des PGPR sur la phénologie des plantes, notamment sur le temps de floraison (Schwachtje *et al.*, 2011; Poupin *et al.*, 2013). Poupin *et al.*, (2013) ont récemment montré que l'inoculation par la PGPR *Burkholderia phytofirmans* PsJN chez *A. thaliana* induit une accélération de la vitesse de croissance et un raccourcissement de la période végétative, amenant ainsi à une floraison précoce des plantes en condition optimale de croissance. Ces changements de phénologie ont été corrélés à une régulation positive des gènes contrôlant la floraison des plantes. Ces résultats mettent en évidence une possible implication des PGPR dans l'échappement des plantes au déficit hydrique mais à notre connaissance aucune étude n'existe de nos jours.

### II.1.2. Les PGPR confèrent de nouvelles capacités aux plantes via des ajustements plastiques

Lors d'un déficit hydrique modéré ou à courte durée, les plantes maintiennent leur statut hydrique par des mécanismes de réduction des pertes d'eau ou de maximisation du prélèvement de l'eau. Il a été communément montré que l'inoculation par des rhizobactéries permet une amélioration du statut hydrique des plantes en condition limitante en eau (pour exemples, Creus *et al.*, 2004; Marulanda *et al.*, 2009; Arzanesh *et al.*, 2011). Outre les modifications de la teneur en eau observées chez les plantes inoculées, les PGPR induisent



des ajustements plastiques conférant de nouvelles capacités aux plantes pour lutter contre les effets négatifs du stress.

Une des actions les plus connues des PGPR est sans aucun doute leur implication dans les changements de morphologie racinaire des plantes (Vacheron *et al.*, 2013). De façon générale, les PGPR induisent une maximisation de la surface d'échange par une augmentation globale du système racinaire (pour exemples, Zahir *et al.*, 2008; Marulanda *et al.*, 2009; Walker *et al.*, 2012). Plus précisément, elles stimulent l'allongement des poils racinaires (Dobbelaere *et al.*, 1999; Contesto *et al.*, 2008; Galland *et al.*, 2012), augmentent le nombre et la taille des racines secondaires (Larcher *et al.*, 2003; Creus *et al.*, 2005; Chamam *et al.*, 2013), mais peuvent réduire la taille de la racine primaire (Dobbelaere *et al.*, 1999). De plus, il a été montré que l'inoculation par la rhizobactérie *Azospirillum* affecte la composition en phospholipides membranaires des racines chez le blé (Bashan *et al.*, 1992), pouvant ainsi jouer sur la plasticité des membranes racinaire. De même, l'inoculation par *Azospirillum lipoferum* CRT1 induit une baisse de la teneur en lignine dans les parois cellulaires de racines du maïs (El Zemrany *et al.*, 2007), et peut faciliter ainsi l'élongation racinaire (Vacheron *et al.*, 2013). Les PGPR peuvent ainsi promouvoir la croissance racinaire et permettre aux plantes d'explorer un volume de sol plus important et ainsi maximiser le prélèvement de l'eau du sol lors d'un déficit hydrique. L'habilité des PGPR à affecter l'architecture racinaire est principalement reliée à leur influence dans la balance hormonale des plantes, notamment dans le rapport entre l'auxine et les cytokinines (Vacheron *et al.*, 2013).

Les PGPR affectent la signalisation ou le niveau de multiples hormones (auxine, cytokinine, ABA, éthylène) *in planta*. De plus, les PGPR produisent également des phytohormones modifiant la concentration rhizosphérique et ainsi interférant dans le statut hormonal des plantes (Dodd *et al.*, 2010). La régulation hormonale via l'action de PGPR, est un point central dans le contrôle de la physiologie, de la croissance et du développement des plantes, pouvant modifier les performances des plantes lors d'un stress.

Lors d'un stress hydrique, les PGPR participent à la réduction des pertes d'eau via la production d'ABA ou des modifications de l'ABA *in planta* (Dodd *et al.*, 2010), hormone clé dans le contrôle stomatique. L'inoculation par *Azospirillum brasilense* chez *A. thaliana* conduit à doubler la concentration d'ABA tissulaire (Cohen *et al.*, 2008). Cette modification de la teneur en ABA a même été observée dans des conditions de stress hydrique. L'inoculation par la souche *Azospirillum lipoferum* induit une augmentation de la concentration en ABA et conduit à un meilleur statut hydrique du maïs lors d'un stress hydrique (Cohen *et al.*, 2009). Les bactéries sont aussi capables de moduler le fonctionnement

physiologique via des modifications de la teneur en ABA. Chez *A. thaliana*, Zhang *et al.* (2008) ont interprété l'augmentation de la vitesse de photosynthèse de plantes inoculées par la bactérie *Bacillus subtilis* comme étant le résultat d'une diminution du niveau d'ABA *in planta* en condition non stressante. Certaines bactéries sont donc capables de modifier les capacités photosynthétiques (Rincon *et al.*, 2008) mais aussi de moduler la teneur en chlorophylle lors d'un stress hydrique (Heidari *et al.*, 2011; Heidari & Golpayegani, 2012; Stefan *et al.*, 2013). L'inoculation simultanée par trois PGPR (*Bacillus cereus* AR156, *Bacillus subtilis* SM21, et *Serratia sp.* XY21) induit un meilleur maintien de la teneur en chlorophylle lors d'un stress hydrique et par conséquent les plantes ont des feuilles plus vertes et présentent moins de symptômes liés au stress (Wang *et al.*, 2012).

En réponse au déficit hydrique, les plantes augmentent la synthèse d'osmo-protecteurs, permettant d'ajuster le potentiel hydrique dans les cellules et de détoxifier les cellules (Farooq *et al.*, 2009). Il a été montré que certaines rhizobactéries peuvent intervenir dans ces réponses en synergie avec les réponses intrinsèques des plantes. L'inoculation chez le riz par des rhizobactéries, surproductrice de glycine-bétaïne, entraînent une amélioration de la résistance des plantes par une augmentation de la matière sèche foliaire et racinaire allant jusqu'à 46% et 80%, respectivement (Yuwono *et al.*, 2005). De même, la co-inoculation avec la souche *Pseudomonas mendocina* Palleroni et un champignon mycorhizien arbusculaire augmentent significativement l'accumulation de proline et l'activité d'enzymes détoxifiantes dont les peroxidases et les catalases, dans les feuilles de laitue lors de stress hydriques modéré et sévère (Kohler *et al.*, 2008). De même, la co-inoculation de plants de concombre par trois différentes PGPR a aussi montré une amélioration de la résistance à la sécheresse notamment par une augmentation de l'activité superoxide dismutase, catalase et peroxidase (Wang *et al.*, 2012). Pareillement, l'inoculation de *Hyoscyamus niger* (jusquiame noire) par deux PGPR *Pseudomonas putida* et *Pseudomonas fluorescens* induit une augmentation de multiple activités enzymatiques impliquées dans la détoxification des ROS dans trois différentes conditions de stress hydrique (Ghorbanpour *et al.*, 2013).

La susceptibilité des plantes au stress hydrique passe aussi par des dommages de la membrane et la composition des lipides (De Paula *et al.*, 2003) amenant à des altérations de la perméabilité des membranes (Palta, 1990). La membrane des cellules constitue une interface dans un système complexe de régulation du statut physiologique des plantes. Les rhizobactéries peuvent induire des changements dans la composition et dans la plasticité des membranes (Bashan *et al.*, 2004; Pereyra *et al.*, 2006), ce qui peut présenter une étape clé dans l'amélioration de la tolérance à la sécheresse.

Il a été montré que les rhizobactéries peuvent aussi moduler la sensibilité des racines et la croissance des feuilles lors d'un déficit hydrique, par modulation de la signalisation de l'éthylène (Glick *et al.*, 1998). L'éthylène joue le rôle de molécule signal lors d'un stress. Les bactéries ont la capacité de réduire la production éthylène par la dégradation de son précurseur l'1-aminocyclopropane-1-carboxylate (ACC) via une activité ACC-déaminase (Belimov *et al.*, 2009). Par exemple, l'activité de l'ACC déaminase de la bactérie *Achromobacter piechaudi* ARV8 a conféré une tolérance à la tomate et le piment lors d'un stress hydrique via une réduction de la production de l'éthylène chez les plantes inoculées (Mayak *et al.*, 2004). Par ailleurs, l'inoculation par *Variovorax paradoxus* 5C-2 a montré une abscission des feuilles matures réduite et retardée chez des plantes ornementales soumises à un stress hydrique par une diminution de la teneur en éthylène (Sharp *et al.*, 2011).

A un niveau transcriptionnel, les rhizobactéries engendrent des modifications de l'expression de gènes liés à la sécheresse. L'inoculation par la bactérie *Paenibacillus polymyxa* provoque chez *Arabidopsis* une induction de l'expression du gène *ERD15*, un gène inductible par le stress hydrique, et du gène *RAB18* inductible par l'ABA (Timmusk & Wagner, 1999).

### II.1.3. Les PGPR permettent d'améliorer la survie des plantes lors d'un déficit hydrique sévère

Il est difficile de séparer les implications des PGPR dans les réponses à l'évitement et à la tolérance à la déshydratation des plantes. Elles impliquent des réponses communes permettant aux plantes de se protéger contre les effets négatifs du stress, et de maximiser leur statut hydrique. Toutefois, quelques études se sont intéressées à l'effet des rhizobactéries sur la survie des plantes lors d'un stress hydrique sévère. Ces études ont montré que l'inoculation par des bactéries transgéniques surproduisant du tréhalose dans leurs cellules, permettent une augmentation de la teneur en tréhalose et un meilleur statut hydrique des plantes, conférant ainsi une meilleure survie au déficit hydrique (Suarez *et al.*, 2008; Rodriguez-Salazar *et al.*, 2009). L'inoculation du haricot par *Rhizobium etli* modifiée, a ainsi permis une survie de 87% des plantes inoculées, contre seulement 7% pour les plantes non inoculées (Suarez *et al.*, 2008). De plus, l'analyse transcriptomique dans les nodules du haricot a montré une régulation positive de gènes impliqués dans la tolérance au stress, et dans le métabolisme du carbone et de l'azote. Le métabolisme du tréhalose a été largement décrit pour son implication dans la tolérance à la sécheresse (Cf Chapitre 5).

L'inoculation par des rhizobactéries induit donc des modifications du développement, de la physiologie et du métabolisme des plantes. L'inoculation par une rhizobactérie n'induit pas la modification d'un seul trait chez les plantes mais entraîne des modifications globales du fonctionnement des plantes. Par exemple, un seul mécanisme n'est pas impliqué dans la stimulation des plantes par *Azospirillum* mais une combinaison de plusieurs mécanismes est impliquée dans ce cas (Bashan & de-Bashan, 2010). En effet, l'inoculation avec *Azospirillum brasilense* induit une augmentation de la teneur en eau et retarde de façon significative la chute du potentiel hydrique, en parallèle avec une augmentation concomitante de la croissance racinaire, de la biomasse aérienne et de la surface foliaire, ainsi qu'une accumulation de proline dans les feuilles et les racines (Casanovas *et al.*, 2002). Cette capacité des PGPR à maximiser les stratégies des plantes, par des voies diverses et en simultané, est un atout réel pour l'amélioration du rendement des cultures.

### III. Le genre *Phyllobacterium* sp. dans les interactions plante-rhizobactéries

La première isolation de *Phyllobacterium* sp. a été rapportée en 1902 par Zimmermann et le nom du genre *Phyllobacterium* a été proposé par Knösel en 1962 pour identifier une bactérie se développant dans des nodules de feuilles de plantes tropicales (*Pavella zimmermannianna* et *Ardisia crispa* ; Mergaert & Swings, 2005). Le genre *Phyllobacterium* appartient à la famille des Phyllobacteriaceae, ordre des Rhizobiales dans la classe des  $\alpha$ - Protéobactéries. Des bactéries du genre *Phyllobacterium* ont été identifiées dans différents environnements et en association plus ou moins étroite avec des végétaux. Elles ont été identifiées dans la rhizosphère de l'épicéa (*Picea abies*) et du lotus (*Lotus spp.* ; Elo *et al.*, 2000; Oger *et al.*, 2004), en étroite connexion avec les racines de la canne à sucre (*Saccharum officinarum* ; Lambert *et al.*, 1990) et la betterave (*Beta vulgaris* ; Lilley *et al.*, 1996). Certaines de ces bactéries ont été caractérisées de bactéries endophytes en interaction avec le trèfle des près (*Trifolium pratense*), le maïs (*Zea mays*), ou encore le cotonnier (*Gossypium hirsutum* ; McInroy & Kloepper, 1995; Hallmann *et al.*, 1997; Sturz *et al.*, 1997). Plus étonnamment, *Phyllobacterium myrsinacearum* a été retrouvée en association avec une micro-algue (Gonzalez-Bashan *et al.*, 2000). L'ensemble de ces études montrent la capacité des *Phyllobacteria* d'interagir avec un large spectre de plantes dans des environnements très contrastés et lui confère un intérêt particulier pour l'étude des interactions plante-rhizobactérie. De plus, l'analyse de sa position taxonomique au sein des autres bactéries a

montré aucun parent pathogène immédiat d'un point de vue phylogénétique (Mantelin *et al.*, 2006b), ce qui est essentiel pour les expérimentations en champs. Leur capacité à stimuler la croissance des plantes n'a été décrite que récemment (Chanway *et al.*, 2000; Bertrand *et al.*, 2001; Larcher *et al.*, 2003).

La souche *Phyllobacterium sp.* STM196 a été isolée en 1997 à partir de broyats de racines de colza (Bertrand *et al.*, 2001), au voisinage d'autres souches du genre *Pseudomonas*, *Vario-vorax* et *Agrobacterium*. Parmi la vingtaine d'isolats sélectionnés lors de cette étude, la souche STM196 (isolat 29-15) s'est révélée la plus efficace pour stimuler la croissance du colza en condition gnotobiotique. Les plantes inoculées avec cette bactérie présentent une augmentation de la matière sèche de 66% et de 52% dans les parties aériennes et racinaires respectivement après 15 jours de croissance des plantules (Bertrand *et al.*, 2001; Larcher *et al.*, 2003). En regard, les souches *Pseudomonas migulae*, *Agrobacterium tumefaciens* et *Variovorax paradoxus* ont respectivement permis un gain de la matière sèche racinaire de 11, 24 et 25%. Ces études ont permis de mettre en évidence le caractère « plant growth-promoting » (PGP) de la souche STM196. Les études menées par l'équipe de Bruno Touraine ont permis de montrer que *Phyllobacterium brassicacearum* STM196 stimule aussi la croissance *in vitro* d' *A. thaliana* et entraîne des changements morphologiques racinaires typiques des PGPR (modifications de l'architecture racinaire : augmentation du nombre, de la longueur des racines secondaires, de la densité et de la longueur des poils absorbants ; Mantelin *et al.*, 2006a; Galland *et al.*, 2012).

Les premières études sur l'interaction entre *Phyllobacterium brassicacearum* et *A. thaliana* ont révélé l'implication de STM196 dans la nutrition azotée des plantes (Mantelin *et al.*, 2006a). Le rôle des PGPR dans la nutrition des plantes est présent chez de nombreuses rhizobactéries (Mantelin & Touraine, 2004). Dans le cas particulier de STM196, il a été montré que l'inoculation permet d'annuler l'inhibition de la croissance des racines secondaires chez *A. thaliana* lors de hautes concentrations en nitrate (Mantelin *et al.*, 2006a). STM196 permet donc une restauration du développement racinaire. Cet effet bénéfique peut être expliqué par une diminution de l'afflux en nitrate et du niveau d'expression de gènes codant des transporteurs de nitrate AtNRT1.1 et AtNRT2.1 dans les racines des plantes inoculées. De plus, deux autres gènes préférentiellement exprimés dans les feuilles, *AtNRT2.5* et *AtNRT2.6* ont été fortement induits lors de l'inoculation. Par la suite, ces deux gènes ont été montrés indispensables à la stimulation de la croissance et aux modifications de l'architecture racinaire chez *A. thaliana* suite à l'inoculation par STM196 (Kechid *et al.*, 2013).

L'implication de STM196 sur le développement racinaire passe aussi par des modifications de la signalisation auxinique (Contesto *et al.*, 2010). Les modifications de l'architecture racinaire via le métabolisme de l'auxine est un mécanisme commun chez la plupart des PGPR (Sukumar *et al.*, 2012). L'inoculation par STM196 induit une augmentation de 50% de la croissance des racines latérales chez *Arabidopsis*. L'abolition de cet effet chez deux mutants affectés dans le transport et la signalisation d'auxine, *aux1* et *axr1*, a montré l'implication de STM196 la signalisation de cette hormone. De plus, l'inoculation par STM196 induit un remaniement de la distribution de l'auxine entre les racines et les feuilles. Les PGPR ont la capacité de sécréter des phytohormones dans leur environnement pouvant interférer avec le fonctionnement intrinsèque des plantes (Dodd *et al.*, 2010). Contesto *et al.*, (2010) ont montré la faible capacité de production d'auxine par STM196 et ont pu ainsi exclure l'action d'auxine exogène sécrétée par STM196.

Enfin, l'implication de l'éthylène a pu être mise en évidence dans les changements de l'architecture racinaire occasionnés par STM196, notamment au niveau des poils racinaires (Contesto *et al.*, 2008; Galland *et al.*, 2012). L'éthylène contrôle l'élongation racinaire chez les plantes (Pitts *et al.*, 1998). L'éthylène joue un rôle important lors des interactions plantes-microorganismes, particulièrement en ce qui concerne le développement racinaire (Ribaudou *et al.*, 2006). De plus, il a été montré que certaines PGPR peuvent moduler la synthèse d'éthylène via la dégradation de son précurseur (Belimov *et al.*, 2009). Contrairement à d'autres PGPR, il a été montré que STM196 affecte l'élongation des poils racinaires, par une activation de la signalisation de l'éthylène, sans affecter la biosynthèse de cette hormone (Galland *et al.*, 2012).

Le cas de *Phyllobacterium brassicacearum* STM196 illustre la complexité et la multiplicité des réponses occasionnées chez les plantes lors de l'inoculation par des PGPR. De plus, l'ensemble des connaissances accumulées sur cette souche en fait un modèle de choix pour étudier le rôle des PGPR dans la réponse d'*Arabidopsis* aux stress abiotiques.

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***Chapitre 2***  
***Matériel et méthodes***  
***&***  
***Développement méthodologique***

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# Matériel et méthodes

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## I. Matériel biologique

Toutes les expériences ont été réalisées sur l'espèce modèle *A. thaliana* (L.) Heynh. L'écotype Columbia (Col-0) a été choisi comme référence. Trois mutants ont été sélectionnés pour l'implication des gènes mutés dans les réponses à l'inoculation et aux stress hydriques. Ces trois mutants par insertion ADN-t sont affectés dans le métabolisme du tréhalose. Deux mutants (*Attre1*<sup>KO</sup> et *Attre1*<sup>OE</sup>) sont affectés dans l'expression du gène d'Arabidopsis (*AtTRE1*) codant l'enzyme de dégradation du tréhalose, la tréhalase (At4G24040.1). La protéine AtTRE1 est localisée dans la membrane plasmique (Frison *et al.*, 2007). Le mutant *Attre1*<sup>OE</sup> (SAIL25C12) est un surexprimeur du gène *AtTRE1*. *Attre1*<sup>OE</sup> provient du fond génétique Col-3, qui est un descendant direct de Col-1, écotype génétiquement très proche de Col-0. Au contraire, le mutant *Attre1*<sup>KO</sup> (SALK147073) est un mutant de l'écotype Col-0, knockout par insertion d'ADN-t dans *AtTRE1*. Le troisième mutant *35S::treF*, fourni par Dr John Lunn (MPI-MP, Golm, Allemagne), exprime constitutivement le gène *TREF* d'*E.coli* codant une trehalase cytoplasmique. Une accession d'*A.thaliana*, Antwerpen (An-1), a été utilisée pour son temps de floraison précoce comparé à Col-0.

La souche bactérienne *Phyllobacterium brassicacearum* STM196 (STM196) a été isolée par J.C Clayet-Marel (UMR113-LSTM) à partir de la rhizosphère du colza (Bertrand *et al.*, 2001).

## II. Méthodes

### II.1. Culture de la souche *Phyllobacterium brassicacearum*

L'équivalent d'une anse de platine a été prélevé d'un stock glycérolé (conservation à -80 °C) puis étalé sur milieu gélosé semi-solide préalablement autoclavé à 120 °C pendant 20 min. La souche *Phyllobacterium brassicacearum* STM196 a été cultivée sur milieu E' (2,9 mM K<sub>2</sub>HPO<sub>4</sub>, 0,8 mM MgSO<sub>4</sub>, 1,7 mM NaCl, 7,9 mM KNO<sub>3</sub>, 0,3 mM CaCl<sub>2</sub>, 0,030 mM FeCl<sub>3</sub>, 3 g l<sup>-1</sup> d'extrait de levure, 10 g l<sup>-1</sup> de mannitol, H<sub>2</sub>O milliQ ; pH 6,8 et 15 g l<sup>-1</sup> d'agar). STM196

a été cultivée en condition semi aérobie dans des boîtes de Pétri de 9 cm de diamètre, scellées au Parafilm (Pechiney Plastic Packaging Company, IL, USA) et placées à l'obscurité à 25 °C.

Après 48h de culture sur milieu solide, une pré-culture a été réalisée à partir d'une colonie isolée prélevée et transférée dans 200 ml de milieu de culture liquide contenu dans un Erlenmeyer stérile. Les Erlenmeyers de pré-culture ont été placés en condition semi-aérobie sous agitation constante (160 rpm ; KS501 digital IKA Labortechnik) à l'obscurité, à 25 °C. La croissance des bactéries en pré-culture a été suivie par densité optique (DO 595 nm). Après croissance des bactéries, la culture s'est effectuée dans un volume plus conséquent destiné à l'inoculation de terre ou de milieu de culture pour plante *in vitro*. Ainsi, des bouteilles contenant 750 ml de milieu liquide, inoculés avec 20 ml de pré-culture bactérienne en phase exponentielle, ont été manipulées dans les mêmes conditions que pour les pré-cultures.

## II.2. Inoculation en terre et in vitro

Les cultures bactériennes en milieu liquide ont été arrêtées pendant la phase exponentielle afin de récupérer les cellules bactériennes par centrifugation (4 000 rpm pendant 10 min à 15 °C). Les culots ont été repris dans de l'eau osmosée avant d'estimer la quantité de cellules par densité optique à 595 nm. Pour toutes les expérimentations réalisées en terre, un mélange (50/50 ; v/v) de terreau et de terre limono-argileuse a été inoculé avec un volume déterminé de façon à obtenir une concentration bactérienne finale d'environ de  $3.10^7$  bactéries par gramme de sol. De manière générale, 20 l de milieu E' inoculé ont été nécessaire pour inoculer 50 kg de terre.

*In vitro*, après centrifugation des cellules bactériennes, le culot bactérien est remis en suspension dans un milieu minéral pour plante (0.5 mM CaSO<sub>4</sub>, 2 mM KNO<sub>3</sub>, 0.5 mM MgCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 Na<sub>2</sub>FeEDTA, 2.5 mM MES, 1X Oligo-élément, H<sub>2</sub>O milliQ, pH 5.7 et de 1.2 % d'agar) afin obtenir une concentration de  $10^8$  cfu ml<sup>-1</sup>.

## II.3. Culture et caractérisation phénotypique des plantes cultivées en sol

Cinq graines d'*Arabidopsis* ont été semées dans des pots de 260 ml contenant 50/50 (v/v) de terreau et de terre limono-argileuse. La terre a été préalablement inoculée et homogénéisée avant le semis. Les graines ont été soumises à stratification en plaçant les pots en chambre froide pendant une durée minimale de 48 h. Les pots ont été ensuite placés dans la plateforme

de phénotypage PHENOPSIS (Granier *et al.*, 2006). La germination a été initiée en disposant les pots à l'obscurité à 17 °C avec un déficit de vapeur d'eau de l'air (VPD) de 0.6 kPa et en vaporisant de l'eau osmosée à la surface des pots 3 fois par jour. Les plantes ont ensuite été cultivées à 12 h de photopériode (190  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  PPFD), 0.8 kPa de VPD et à une température de 20 °C jour /17 °C nuit. L'humidité du sol a été maintenue par application d'eau osmosée par spray 3 fois par jour jusqu'à une taille suffisante de la rosette (stade 1.02, Boyes *et al.*, 2001) pour supporter une irrigation plus abondante.

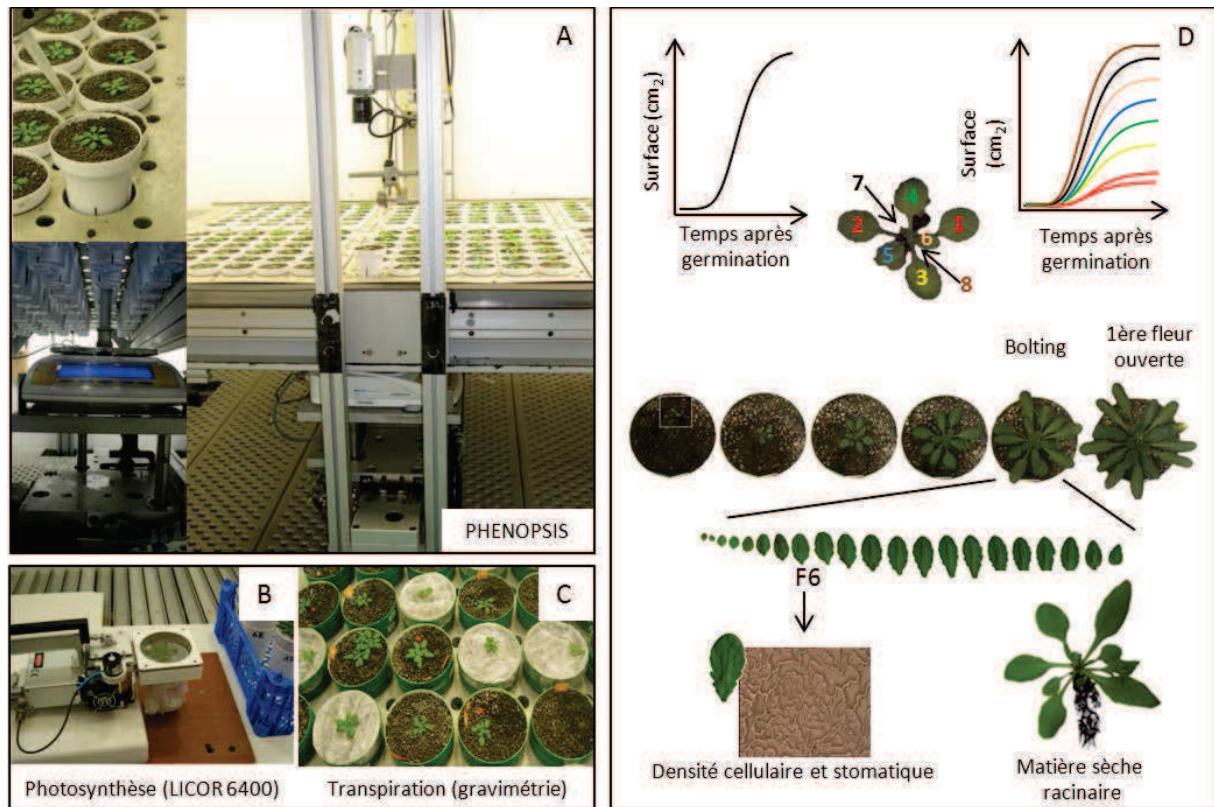
### II.3.1. Application de différents déficits hydriques

L'humidité relative du sol ( $\text{HR}_{\text{sol}}$ , exprimée en  $\text{g H}_2\text{O g}^{-1} \text{sol sec}$ ) initiale a été calculée grâce à la perte d'eau d'échantillons de terre placés à l'étuve (60°C). Le stress hydrique a été appliqué à partir l'apparition des deux premières feuilles (stade 1.02, Boyes *et al.*, 2001) en arrêtant l'irrigation du substrat de culture jusqu'à atteindre l'humidité relative souhaitée. Dans le substrat utilisé, une humidité pondérale inférieure à 30% (0.30  $\text{g H}_2\text{O g}^{-1} \text{sol sec}$ ) affecte significativement le développement d'*A. thaliana* (Granier *et al.*, 2006); l'humidité contrôle du sol a ainsi été fixée à 35%. L'humidité du sol de chaque pot a été maintenue pendant toute la durée de l'expérimentation par pesées et réajustement réguliers du contenu en eau de chaque pot par arrosage avec une solution nutritive de Hoagland diluée au 10<sup>ème</sup> (Hoagland & Arnon, 1950), par le robot de phénotypage haut-débit PHENOPSIS (Fig. 1A) ou de façon manuelle. Les pots soumis à un stress hydrique modéré continu ont été privés de solution nutritive jusqu'à atteindre 20%  $\text{HR}_{\text{sol}}$ , valeur conservée jusqu'à la récolte. Les pots soumis à un stress hydrique sévère ponctuel ont été privés d'irrigation jusqu'à atteindre une  $\text{HR}_{\text{sol}}$  souhaitée (de 10 à 6%  $\text{HR}_{\text{sol}}$ ), seuil à partir duquel les pots ont été à nouveau irrigués pour atteindre progressivement l'humidité contrôle de 35%. Le potentiel hydrique du sol a été déterminé pour les différentes  $\text{HR}_{\text{sol}}$  (WP4-T dewpoint meter, Decagon Devices, Pullman, WA, USA).

### II.3.2. Caractérisation phénotypique des plantes cultivées en terre

#### *Expansion de la rosette, production dynamique des feuilles et phénologie*

Pendant la culture, des images zénithales des plantes ont été prises au cours du temps (grâce à la caméra du robot de phénotypage PHENOPSIS ; PROSILICA AVT GC 1600C camera, ALLIED, Stradroda, Germany ; Fig. 1A) afin de déterminer la dynamique de croissance des



**Fig.1. L'automate de phénotypage PHENOPSIS de la station 'Montpellier Plant Phenotyping Platform' (M3P) et le phénotypage des plantes. (A)** Grâce à un bras amovible PHENOPSIS permet de peser, irriguer précisément et prendre une image verticale et latérale, sur plus de 504 plantes d'*Arabidopsis thaliana* dans des conditions environnementales rigoureusement contrôlées. **(B)** Mesure de la photosynthèse grâce à la chambre plante entière *Arabidopsis* connectée au système d'analyseur de gaz (LI-6400XT; LI-COR, Inc., Lincoln, NE). **(C)** Mesure de la transpiration des plantes par gravimétrie. Les plantes sont entourées au collet par 4 couches de film plastique afin d'empêcher les pertes d'eau par le sol. **(D) haut :** analyse de la croissance des plantes à l'échelle de la rosette ou des feuilles individuellement ; **milieu :** récolte des plantes au stade bolting ou à 1<sup>ère</sup> fleur ouverte. La surface individuelle de chaque feuille est évaluée par ordre de croissance ; **bas :** une empreinte épidermique de la feuille 6 permet l'évaluation du nombre de cellules et de stomate. La biomasse sèche des racines est mesurée après nettoyage du système racinaire à l'eau osmosée.

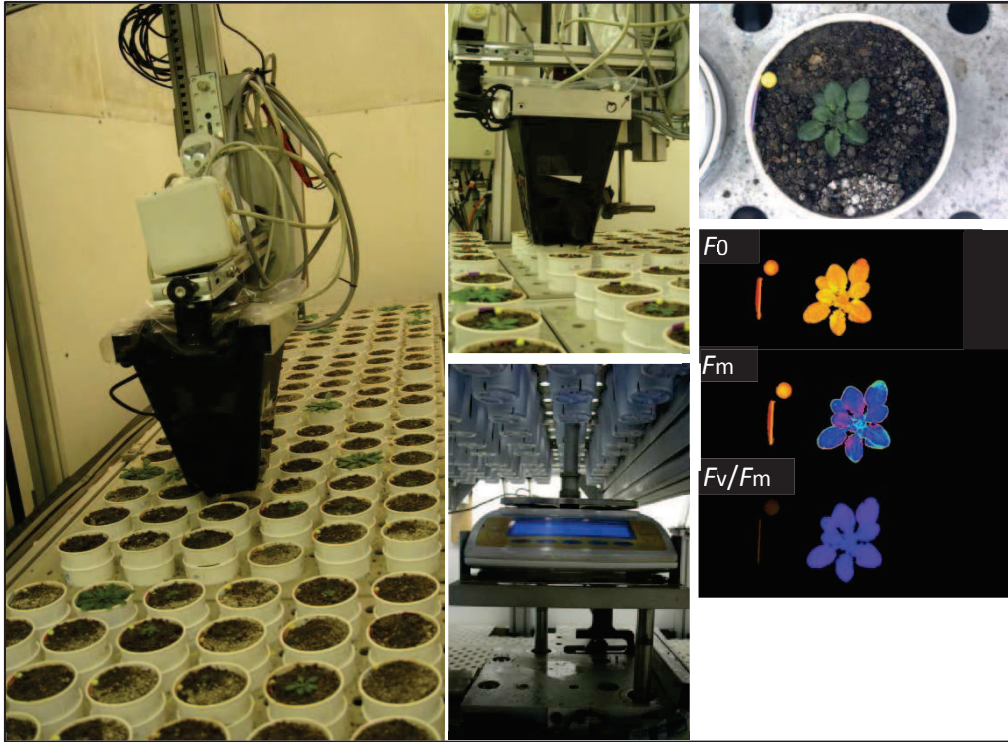
rosettes (surface projetée des rosettes ;  $RA_{proj}$ ) ou des feuilles individuelles à l'aide d'un logiciel d'analyse d'images (ImageJ 1.43C ; Fig. 1D).

Une courbe de croissance sigmoïdale a été ajustée pour chaque plante selon l'équation  $RA_{proj} = a / (1 + \exp(-(d-a/2)/b))$  où  $a$  est la surface projetée maximale,  $d$  est le nombre de jours après semis, et,  $b$  la pente. La vitesse maximale de l'expansion foliaire ( $R_{max}$ ,  $\text{mm}^2 \text{d}^{-1}$ ) a été calculée de la dérivée d'ordre 1 du modèle logistique à  $d_0$  comme  $R_{max} = a/(4b)$ . La durée (jours) de l'expansion de la rosette a été estimée comme le temps pour que la rosette augmente de 5 à 95% de la surface maximale selon  $d_0 - b \ln((1/0.95) - 1)$ .

Le nombre de feuilles initiées, visibles à l'œil nu, a été compté tous les 2-3 jours afin de déterminer le phyllochrone (j feuille<sup>-1</sup>) de chaque plante après germination. Le temps pour atteindre l'apparition des boutons floraux (« bolting ») et le temps de floraison ont respectivement été déterminés comme le nombre de jours depuis la germination jusqu'à l'apparition macroscopique des bourgeons floraux (stade 5.01, Boyes *et al.*, 2001) et à première fleur ouverte (stade 6.00).

### *Traits plantes entières et foliaires*

Les plantes ont été récoltées à deux stades de développement selon les expérimentations : à l'apparition des bourgeons floraux et à première fleur ouverte (Fig. 1D). La masse fraîche (MF, mg) de la rosette a été déterminée immédiatement après retrait de l'inflorescence, puis chaque rosette a été placée entre deux feuilles de papier absorbant humides dans une boîte de Petri stockée à l'obscurité à 4 °C pendant 24 h. Après réhydratation, la masse fraîche réhydratée ( $MF_{réhydratée}$ , mg) de la rosette a été déterminée, puis la hampe florale, les limbes et les pétioles ont été pesés séparément. Les limbes et les pétioles ont été disposés sur une feuille de papier et scannés (Fig. 1D). Tous ces organes ont été mis à l'étuve séparément pendant 1 semaine à 60 °C afin de déterminer leur masse sèche (MS, mg). La surface totale de la rosette (SF,  $\text{cm}^2$ ) a été calculée par la somme des surfaces de chaque limbe mesurée grâce à ImageJ (ImageJ 1.43C). La surface spécifique de la rosette (SFF,  $\text{cm}^{-2} \text{g}^{-1}$ ) a été calculée selon le rapport de la surface totale et de la masse sèche des limbes. La surface massique a été calculée comme l'inverse de la SFF. La teneur relative en eau (TER) des limbes a été calculée selon le rapport  $(MF - MS) / (MF_{réhydratée} - MS)$ , où MF et MS sont respectivement la masse fraîche et la masse sèche des limbes. La teneur en matière sèche des feuilles (TMSF,  $\text{mg g}^{-1}$ ) a été calculée par le rapport  $MS / MF_{réhydratée}$  des limbes. Une estimation de l'épaisseur moyenne des limbes (E,  $\mu\text{m}$ ) a été calculée par le rapport  $MF_{réhydratée} / SF$  (Vile *et al.*, 2005). Une empreinte épidermique de la 6<sup>ème</sup> feuille a été réalisée au moment de la récolte par application



**Fig.2. Mesure de la fluorescence de la chlorophylle par l'automate de phénotypage PHENOPSIS.** Le rendement de fluorescence minimal des feuilles ( $F_0$ ) et le rendement maximal des feuilles de la rosette ( $F_m$ ) ont été mesurés sur des plantes adaptées à l'obscurité. A partir de ces paramètres, le rendement quantique maximum de la photochimie du PSII a été déterminé selon le rapport  $(F_m - F_0)/F_m = F_v/F_m$ .

d'une couche de vernis incolore sur l'épiderme supérieur de la feuille. L'empreinte a été analysée au microscope (Leitz DM RB; Leica, Wetzlar, Germany) à l'aide du logiciel d'analyse d'image Optimas (BioScan-Optimas 4.10, Edmond, WA, USA ; Fig. 1D). La densité des cellules (DC, mm<sup>2</sup>) et la densité des stomates (DS, mm<sup>2</sup>) ont été obtenues en mesurant deux zones de 0,04 mm<sup>2</sup> en haut et en bas de la feuille. L'indice stomatique (IS) a été calculé comme étant le pourcentage de stomates sur le nombre total de cellules (IS = nb stomates / (nb stomates + nb cellules)).

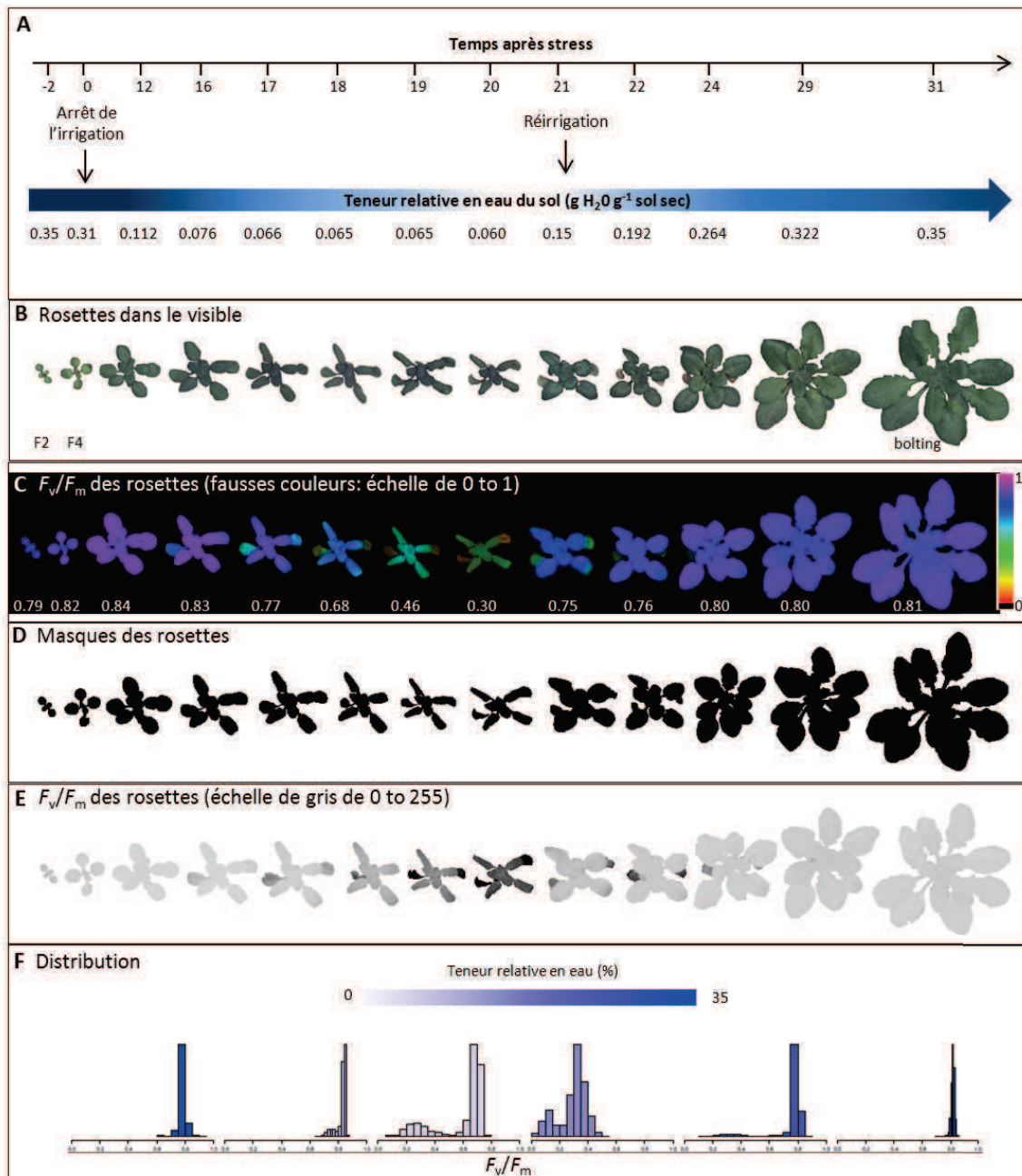
### *Fluorimétrie : mesures de l'efficacité de l'appareil photosynthétique du Photosystème II*

Des mesures de fluorescence du photosystème II ont été réalisées par imagerie (Imaging-Pam MAXI Version M-Series ; caméra CCD W-IMAG-K4, Walz, Germany) sur les plantes soumises à un stress hydrique fort. Les analyses d'images (Imagin Win, v.2.40b) ont permis d'obtenir le rendement de fluorescence minimal des feuilles adaptées à l'obscurité ( $F_0$ ) et le rendement maximal des feuilles de la rosette adaptées à l'obscurité ( $F_m$ ; Fig. 2). A partir de ces paramètres, le rendement quantique maximum de la photochimie du PSII a été déterminé selon le rapport  $(F_m - F_0)/F_m = F_v/F_m$ . Ce rapport est utilisé comme un indicateur de l'état de la plante ; une plante saine ayant un rapport  $F_v/F_m$  proche de 0.80 (Woo *et al.*, 2008). Les valeurs de  $F_v/F_m$  ont été suivies au cours du dessèchement du sol. Le développement d'une macro ImageJ a permis de récupérer les valeurs de chaque pixel composant la rosette et d'analyser les distributions de pixels dans les rosettes (Fig. 3).

### *Transpiration et échanges gazeux des plantes*

Les échanges gazeux ont été déterminés au bolting, i.e. juste avant la récolte. La transpiration des plantes a été mesurée par gravimétrie sur une période d'environ 3 jours et 3 nuits par pesées successives des pots toutes les 3 h approximativement. Les plantes ont été entourées au collet par 4 couches de film plastique afin d'empêcher les pertes d'eau par le sol (Fig. 1C). La vitesse de transpiration (mg H<sub>2</sub>O h<sup>-1</sup>) a été estimée par la pente de la régression linéaire entre la masse des pots et le temps indépendamment pour les périodes nocturnes et diurnes. La surface projetée des rosettes (cm<sup>2</sup>) a été déterminée chaque jour à partir de photographies zénithales comme précédemment décrit. La vitesse de transpiration a alors été exprimée par unité de surface projetée (mg H<sub>2</sub>O h<sup>-1</sup> cm<sup>-2</sup>) en utilisant la surface du jour correspondant. L'efficacité d'utilisation de l'eau (EUE, cm<sup>2</sup> g<sup>-1</sup> H<sub>2</sub>O), quantité de matière sèche synthétisée par unité d'eau perdue, a été estimée de manière non destructive par la vitesse d'expansion de





**Fig.3. Récupération des valeurs de pixels  $F_v/F_m$  au cours de la mise en place du stress hydrique et de la reprise de croissance après réirrigation.** (A) Correspondance entre le temps après stress et la teneur en eau du sol. (B) Images dans le visible de la croissance d'une rosette. (C)  $F_v/F_m$  des rosettes représentées par des fausses couleurs avec une échelle de 0 à 1 (échelle du logiciel Imaging-Pam MAXI Version M-Series, Walz, Germany). (D) Masques des rosettes créés par analyse des images dans le visible par macro développée sous Image J (Rasband, Bethesda, Maryland, USA). (E) Création d'une échelle de gris (de 0 à 255) par calcul des images  $F_0$  et  $F_m$ . (F) Distributions des valeurs de pixels  $F_v/F_m$ .

la rosette (VER,  $\text{cm}^2 \text{d}^{-1}$ ) et par la vitesse de transpiration. La vitesse d'expansion de la rosette estimée à partir de l'analyse d'images zénithales a été convertie par unité de matière sèche selon la surface massique.

La vitesse d'assimilation du  $\text{CO}_2$  (photosynthèse) a été mesurée sur les mêmes plantes grâce à la chambre plante entière *Arabidopsis* connectée au système d'analyseur de gaz (LI-6400XT; LI-COR, Inc., Lincoln, NE; Fig. 1B). Les flux de carbone ( $\mu\text{mol s}^{-1} \text{cm}^{-2}$ ) ont été déterminés à un état stable (approximativement 30 min après allumage et extinction des lampes) à  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD,  $20^\circ\text{C}$  et à 350 ppm de  $\text{CO}_2$ . Toutes ces mesures ont été effectuées dans les mêmes conditions de culture que pour la croissance des plantes.

#### *Dosage des sucres solubles et de l'acide abscissique des parties aériennes*

Les rosettes ont été récoltées au stade bolting, en milieu de journée, et immédiatement congelé dans de l'azote liquide. La teneur en sucres solubles (fructose, glucose, saccharose ;  $\mu\text{mol FM}$ ) a été déterminée selon Hummel *et al.* (2010). Le dosage d'acide abscissique (ABA) a été réalisé selon Quarrie *et al.* (1988) comme précédemment décrit dans Barrieu and Simonneau (2000): des extraits aqueux ont été obtenus après broyage et traitement des parties aériennes au bain marie à  $70^\circ\text{C}$  pendant 5 min ( $5 \text{ ml mg}^{-1}$ ) suivie d'une agitation à  $4^\circ\text{C}$  durant une nuit. Les extraits ont été ensuite centrifugés à  $16\,000 \text{ g}$  pendant 10 min à  $4^\circ\text{C}$ , le surnageant a été conservé à  $-20^\circ\text{C}$ . La teneur en ABA des feuilles ( $\text{ng ABA g}^{-1}$  de matière fraîche) a été déterminée par un dosage radio-immunologique en utilisant un anticorps monoclonal.

#### *Dosage du tréhalose, tréhalose-6-phosphate et autres métabolites*

##### *Extraction du tréhalose et du T6P*

Les rosettes ont été récoltées au stade bolting, en milieu de journée, et immédiatement congelé dans de l'azote liquide. Le tréhalose et le tréhalose-6-phosphate (T6P) ont été extraits et dosés selon les protocoles déjà décrits dans Lunn *et al.* (2006) et ont été réalisés au MPI-MP à Golm (Allemagne). Les rosettes ont été broyées à froid grâce à un vibro-broyeur réglé à 30 secousses/s pendant 30 s. Des aliquots (18-20 mg) de matériel congelé ont été transférés dans des tubes à bouchon vissé de 2 ml et ont été extraits, dans l'azote liquide, par addition de  $350 \mu\text{l}$  de  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (Chloroforme/Methanol ; 3/7, v/v ; eau glacée). Après 2 h d'incubation à  $-20^\circ\text{C}$ ,  $200 \mu\text{l}$  d'eau glacée ont été ajoutée aux échantillons qui sont ensuite centrifugés pendant 10 min à  $4^\circ\text{C}$  ( $14\,000 \text{ rpm}$ ). La phase aqueuse supérieure  $\text{CH}_3\text{OH}$  a été

transférée dans un nouveau tube de 1.5 ml placé dans la glace. La phase inférieure  $\text{CHCl}_3$  et le culot ont été ré-extraits par addition de 300  $\mu\text{l}$  d'eau glacée. Après mélange et centrifugation, comme décrit précédemment, la deuxième phase supérieure a été additionnée à la première. Les extraits ont été stockés à  $-20\text{ }^\circ\text{C}$  pendant la nuit. Le solvant a été ensuite évaporé en utilisant un centrifugal vacuum dryer à  $38\text{ }^\circ\text{C}$  pendant 5 h (SpeedVac SC 110A Concentrator, Savant), et le résidu a été dissout dans 350  $\mu\text{l}$  d'eau glacée, centrifugé ( $4\text{ }^\circ\text{C}$ , 1 min, 14 000 rpm) et conservé à  $-80\text{ }^\circ\text{C}$ . Un aliquot de 125  $\mu\text{l}$  a été déposé sur une plaque (MultiScreen Filter plates with Ultracel-10 Membrane) et centrifugés à  $15\text{ }^\circ\text{C}$  pendant 90 min à 2500 rcf afin d'enlever les composants à hauts poids moléculaire. Le filtrat a été transféré dans un nouveau tube de 1.5 ml à bouchon vissé, et conservé à  $-80\text{ }^\circ\text{C}$ .

### *Dosage du tréhalose*

Le dosage du tréhalose est un dosage fluorométrique suivant la dégradation du tréhalose en glucose par une tréhalase bactérienne sur des échantillons dépourvu de leur contenu en glucose. La détermination de la teneur en tréhalose est alors effectuée par comparaison avec des échantillons standards dont la concentration en tréhalose est connue. 5  $\mu\text{l}$  d'extraits ont été placés dans une plaque de microtitration 96 puits opaque et ont été incubés avec un mix Catalase/Glucose oxydase (30 KU/ml ; 20 KU/ml) dans un volume final de 35  $\mu\text{l}$  contenant 50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.5, 1 mM  $\text{MgCl}_2$  10 mM NaCl et 835  $\mu\text{L}$  d'eau. La réaction contrôle (blancs) de chaque échantillon a été réalisée par l'ajout de 0.02 U de la tréhalase d'*Escherichia coli* à la préparation précédemment utilisée. Après incubation à  $30\text{ }^\circ\text{C}$  pendant 60 min à agitation constante (1250 rpm), la réaction est stoppée en augmentant la température à  $80\text{ }^\circ\text{C}$  pendant 15 min puis la plaque de microtitration est ramenée à  $4\text{ }^\circ\text{C}$ . Les échantillons standards (0-40 pmol de tréhalose) sont alors rajoutés aux échantillons précédemment soumis à la tréhalase. La détermination du tréhalose est alors réalisée par l'ajout d'un mix (55  $\mu\text{l}$ ) : 4 U de glucose oxydase, 0.05 U de peroxydase du raifort (HRP), 0.02 de tréhalase et 83.3  $\mu\text{l}$  du réactif Amplex Red reagent (Invitrogen Molecular Probes) dans 50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.5, 1 mM  $\text{MgCl}_2$  et 10 mM NaCl. Après une agitation légère, la réaction a été suivie en utilisant un lecteur de plaque de microtitration Synergy HT en mode fluorescence avec une longueur d'onde d'excitation de 530 nm et d'émission de 590 nm jusqu'à la stabilisation de la pente (30-45 min). La vitesse de réaction a été calculée automatiquement grâce à l'utilisation du logiciel KC4 (Bio-Teck), et la teneur en tréhalose de chaque échantillon a été déterminée par comparaison de la vitesse de réaction des échantillons standards.

*Dosage du tréhalose-6-phosphate et autres métabolites*

Le dosage du T6P a été réalisé sur les mêmes échantillons utilisés pour le dosage du tréhalose, grâce un système Dionex HPLC (LC ; Sunnyvale, CA, U.S.A), couplé à un spectromètre à masse triple quadripôle Finnigan TSQ Quantum (MS-Q3, ThermoFinnigan, Waltham, Ma, U.S.A). 100  $\mu$ l d'extraits ont été passés dans une pré-colonne (Dionex) 2x50 mm AG11-HC, puis les composés anioniques ont été séparés par une colonne (Dionex) 2x250 mm IonPac AS11-HC à 25°C comme décrit dans Lunn *et al.* (2006). L'éluât est directement dirigé vers le MS-Q3, qui a été utilisé dans un mode de suivi de réactions multiples, avec une source d'ionisation par électro-pulvérisateur en mode d'ionisation négative. La largeur des pics Q1 et Q3 ont été de 0.5 et 0.7 m/z respectivement. Le T6P a été sélectionné en utilisant un ion parent de 421.1 m/z dans le premier quadripôle, et un ion produit de 79 m/z dans le troisième quadripôle. Les métabolites ont été quantifiés par comparaison avec une courbe de calibration utilisant des standards authentiques.

*II.4. Culture et caractérisation phénotypique des plantes cultivées in vitro*

La surface des graines a été stérilisée dans une solution contenant 4% de javel additionnée de quelques gouttes de Tween 20 pendant 10 min sous agitation constante. Les graines ont été ensuite rincées trois fois avec de l'eau milliQ stérile, et semées à raison d'une cinquantaine par boîte de Pétri carrée de 12 cm de côté contenant 43.5 ml de milieu de culture minérale semi-solide préalablement autoclavé à 120 °C pendant 20 min. Ce milieu est composé de 0.5 mM CaSO<sub>4</sub>, 2 mM KNO<sub>3</sub>, 0.5 mM MgCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05 Na<sub>2</sub>FeEDTA, 2.5 mM MES, 1X Oligo-élément, H<sub>2</sub>O milliQ, pH 5.7 et de 1.2 % d'agar. Les boîtes ont été ensuite placées à l'obscurité à 4 °C pendant au minimum 48 h avant d'être placées verticalement dans une chambre de culture en jours longs (16 h de jour ; 21 °C ; 20 000 lux). Après sept jours de culture en chambre, les plantules ont été transférées (4 ou 5 par boîtes) sur un milieu de culture inoculé ou non par une solution bactérienne de manière à obtenir une concentration finale de 10<sup>8</sup> cfu ml<sup>-1</sup>. Après sept jours de croissance sur un milieu inoculé ou non (14 jours de croissance à partir du semis), les boîtes de culture ont été scannées et analysées grâce au logiciel ImageJ (Rasband, Bethesda, Maryland, USA) avec le module d'extension NeuronJ 1.01 (Meijering *et al.*, 2004). La longueur de la racine primaire a ainsi été déterminée. La masse fraîche et la masse sèche des feuilles et des racines ont été indépendamment mesurées.

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## ***Chapitre 2***

# ***Développement méthodologique***

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## Focus Article



# Phenotyping the kinematics of leaf development in flowering plants: recommendations and pitfalls

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Leaves of flowering plants are produced from the shoot apical meristem at regular intervals and they grow according to a developmental program that is determined by both genetic and environmental factors. Detailed frameworks for multiscale dynamic analyses of leaf growth have been developed in order to identify and interpret phenotypic differences caused by either genetic or environmental variations. They revealed that leaf growth dynamics are non-linearly and nonhomogeneously distributed over the lamina, in the leaf tissues and cells. The analysis of the variability in leaf growth, and its underlying processes, has recently gained momentum with the development of automated phenotyping platforms that use various technologies to record growth at different scales and at high throughput. These modern tools are likely to accelerate the characterization of gene function and the processes that underlie the control of shoot development. Combined with powerful statistical analyses, trends have emerged that may have been overlooked in low throughput analyses. However, in many examples, the increase in throughput allowed by automated platforms has led to a decrease in the spatial and/or temporal resolution of growth analyses. Concrete examples presented here indicate that simplification of the dynamic leaf system, without consideration of its spatial and temporal context, can lead to important misinterpretations of the growth phenotype. © 2013 Wiley Periodicals, Inc.

#### How to cite this article:

*WIREs Dev Biol* 2013. doi: 10.1002/wdev.119

## INTRODUCTION: HOW DO SHOOTS OF DETERMINATE PLANTS DEVELOP?

The processes by which aboveground vegetative tissues of determinate flowering plants are

established and affected by environmental and genetic factors have been widely studied in many species. Shoot establishment results from the combination of developmental changes, i.e., the succession of events that contribute to the increase in organ number

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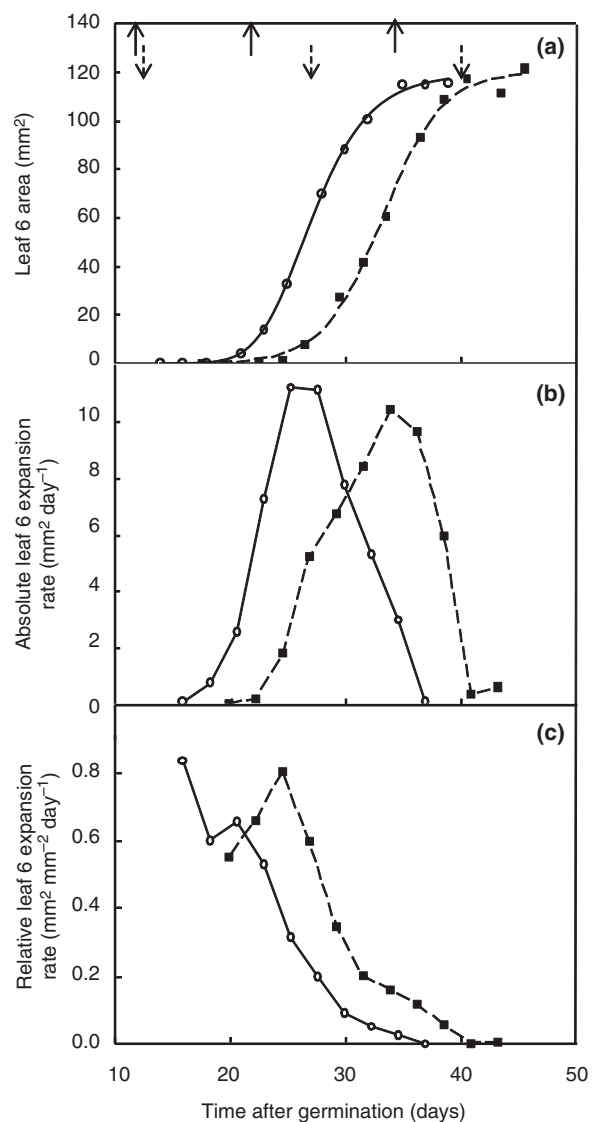
<sup>†</sup>These authors equally contributed to the article.

Conflict of interest: The authors have declared no conflicts of interest for this article. Additional Supporting Information may be found in the online version of this article.



with growth, i.e., the irreversible increase in organ dimensions over time. Developmental changes are often abrupt and take the form of a series of discrete events such as leaf initiation, emergence, or end of expansion (Figure 1(a), see also refs 1 and 2 for other developmental changes at the whole plant scale). In contrast, growth, such as the dynamic change in leaf area is continuous (Figure 1(a), see also refs 3, 4 for other variables such as leaf thickness or cell density). Soon after germination, leaves are initiated on the shoot apical meristem and remain macroscopically small and hidden during the first period (Figure 1(a)). Floral transition in the meristem coincides with the cessation of leaf initiation. Environmentally and genetically induced changes in the timing of floral transition have been extensively studied in a number of species and nearly all of the conditions that affect this timing also alter the total number of leaves.<sup>5–8</sup> At the individual leaf level, the leaf grows at a maximal relative expansion rate, i.e., a maximal area formed per unit area and unit of time, during the hidden phase (Figure 1(c)), whereas absolute leaf expansion rate, i.e., the area formed per unit of time, is low during this phase (Figure 1(b)).<sup>9</sup> Upon emergence, relative leaf expansion rate decreases over time until the cessation of expansion, whereas absolute leaf expansion rate increases until a maximal value and decreases afterwards.<sup>10</sup> These trends are similar in eudicots and monocots with differences in the spatial distribution of expansion rate over the lamina resulting in a long phase with linear expansion, i.e., constant absolute expansion rate in monocots which does not occur in eudicots (see ref 11 for details). When dynamics of expansion are compared among successive leaves of a same plant, it appears that they grow at different rates and that the whole duration of shoot leaf area expansion may also differ.<sup>12,13</sup> The end of plant leaf development coincides with late events related to plant reproduction and finally senescence. The accuracy of quantitative description of both developmental and growth changes depends on the frequency of variable measurement over the considered period, i.e., from germination to reproduction if the whole plant cycle is considered. Low-frequency measurements might result in missing meaningful ruptures and subtle variations in trends.

Growth and development of multicellular organisms are characterized by a complex coordination of cell division and cell expansion.<sup>14–18</sup> In plants, these cellular processes have been tracked over time and spatially over simple leaves. Detailed kinematic analyses have revealed complex and tightly controlled spatial and temporal patterns of cell division and



**FIGURE 1** | The same final leaf area can be reached via different dynamics and durations of developmental phases. Dynamics of leaf 6 area (a) and corresponding changes in absolute (b) and relative (c) leaf expansion rate for two *Arabidopsis thaliana* genotypes, Ler (○) and *elo-1* (■) ( $n = 5$  or  $6$ ). The curves fitted in (a) are 3 parameter sigmoids for Ler  $y = 116.56 / (1 + \exp^{-((t-17.11)/2.25)})$  and *elo-1*  $y = 120.22 / (1 + \exp^{-((t-23.18)/2.58)})$ . For each genotype, leaf development was characterized by three successive stages shown in (a): dates of initiation, emergence, and end of expansion. They are indicated from left to right by solid upward and dashed downward arrows for Ler and *elo-1*, respectively. Note that the two genotypes reach the same final leaf 6, but with different dynamics of leaf expansion. The longer duration of expansion for *elo-1* is due to a longer phase between leaf initiation and leaf emergence (the hidden phase), whereas the duration of the phase between leaf emergence and end of expansion (the visible phase) did not significantly differ between the two genotypes. The figure is adapted from the dataset used in ref 22. Growth conditions and genotypes are described in Supplementary Table 1. Methods are given in Supplementary Information 1 and 2.

cell expansion and how they are modified in genetic variants or upon environmental changes.<sup>10,19–24</sup> Correlations between molecular data and kinematically determined growth parameters with similar spatial and temporal resolution have given insights into the genetic networks controlling cell proliferation and growth.<sup>25,9</sup> However, most kinematic data that are necessary to quantify spatial and temporal changes in growth and underlying cellular processes are obtained from manual measurements that are time-consuming, destructive, and generally laborious. As a consequence, all the detailed spatial and/or temporal growth analyses have been performed at low throughput, on a limited number of genotypes, i.e., a wild-type with a knock-out mutant and/or an over-expressor line, or in a limited number of scenarios, i.e., one genotype grown with and without a constraint.

### FROM LOW TO HIGH-THROUGHPUT LEAF GROWTH ANALYSES: A TENDENCY TOWARDS SIMPLIFICATION OF THE DYNAMIC LEAF SYSTEM

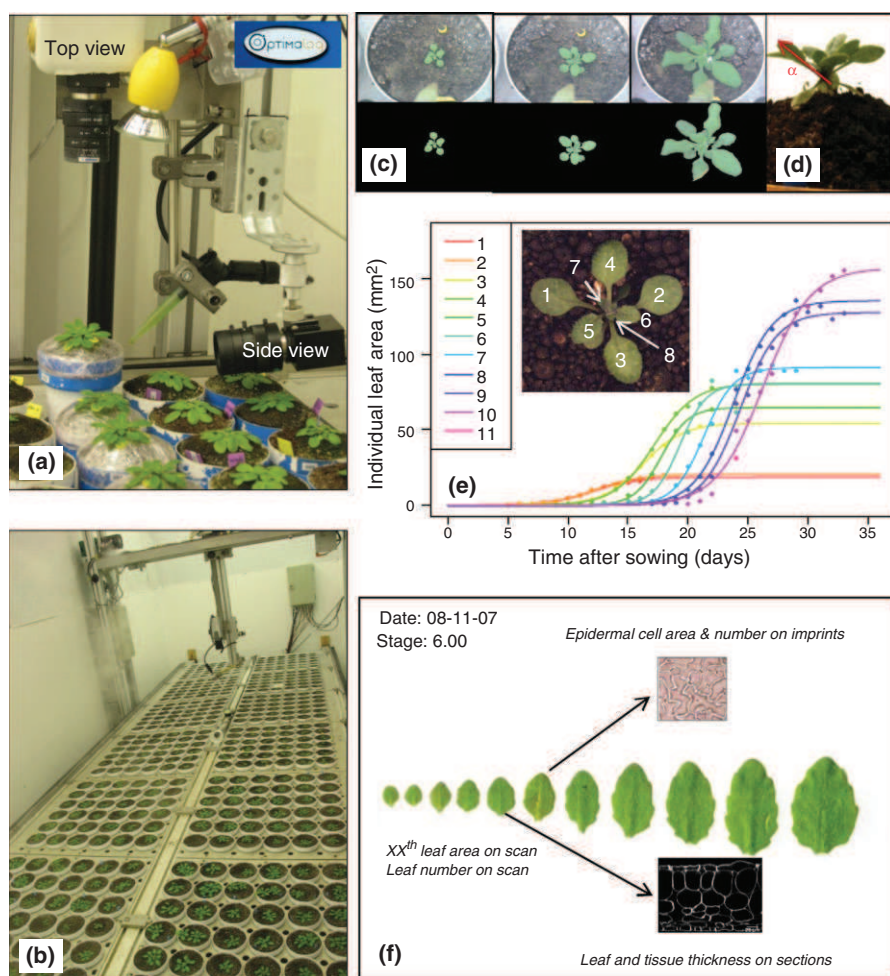
Automated platforms have been developed in many groups allowing the culture of a high number of plants, i.e., hundreds to thousands, in greenhouses or growth-chambers.<sup>26–32</sup> Plants grown in these platforms are either imaged or their aerial organs are attached to displacement transducers for the nondestructive measurement of shoot growth (see Figure 2, <http://www.plantaccelerator.org.au/> and <http://bioweb.supagro.inra.fr/phenodyn/>) as examples). Because of its small size, short life cycle, and the genetic resources available in different stock centers, *Arabidopsis thaliana* has been selected as a model plant for the systematic characterization of growth and development.<sup>33,34</sup> Many platforms are now devoted to shoot growth phenotyping in this species (Figure 2(a)–(e)).<sup>26,27,29,30</sup> Image-processing techniques are used to integrate sequences of 2D plant organ images, issued from top-down imaging, over time with the aim to extract useful quantitative traits and compare genotypes on a robust dynamic basis (Figure 2(a)–(c)).<sup>29,31,35,36</sup> The circadian timing at which growth measurements are performed is important when analyzing growth with 2D images, such as *A. thaliana* rosette expansion with pictures from above. The diurnal hyponastic leaf movements can introduce artifacts in such measurements if care is not taken to time them right.<sup>37–40</sup> In addition, in certain environmental conditions such as high temperature or for certain genotypes, rosette area

measured from above is biased because of leaf twisting, hyponasty, or overlap.<sup>29,41</sup> In these systems, leaf angles can be corrected for by an extra side-view camera (Figure 2(a) and (d)).<sup>41</sup> But, in general, it is argued that overlap between leaves is not considered as an issue because the rosette area imaged from the top is also the most active surface in photosynthesis.

Large-scale phenotyping initiatives have been launched in high-throughput platforms with different purposes including the identification of genotypes with larger rosette area in optimal or stress conditions, the identification of alleles controlling rosette area and its plasticity, and the functional analyses of targeted genes controlling rosette area.<sup>42–46</sup> In most studies, adding manual information or destructive measurements (Figure 2(f)) to the automated recorded data have helped clarifying the circuitry that links the different levels of growth organization, from whole plant leaf area down to leaf production, individual leaf area or shape, cell division, cell expansion, physiological modules, and molecules.<sup>29,36,43,45–47</sup> In all these examples, increasing the throughput of growth analyses to hundreds of plants has been made possible and has gained a deeper understanding of how shoots develop and what are the genes and the processes that contribute to growth control. However, phenotypic analyses in this high-throughput context were very simplified compared to the kinematic leaf growth analyses reported previously, i.e., either by recording easily accessible and automatically measurable traits or by limiting the temporal and/or spatial resolutions of the analyses. In the following sections, specific examples illustrate that recording phenotypic traits outside of temporal and spatial contexts can lead to biased conclusions on growth phenotypes. This article focus on examples issued from the comparison of leaf growth phenotypes in datasets obtained for *A. thaliana*. However, concepts and issues illustrated here can be easily translated to other determinate flowering plants. In addition, in all following examples, leaf growth phenotypes were compared in different genotypes grown together in a same experiment, i.e., a same environment to reveal the ‘genotype effect’. Examples are taken from our group, combining unpublished and published datasets which were re-analyzed and are systematically compared to results obtained by others.

### PHENOTYPING LEAF NUMBER OR SIZE AT A GIVEN DATE: WHICH CONCLUSION CAN BE DRAWN?

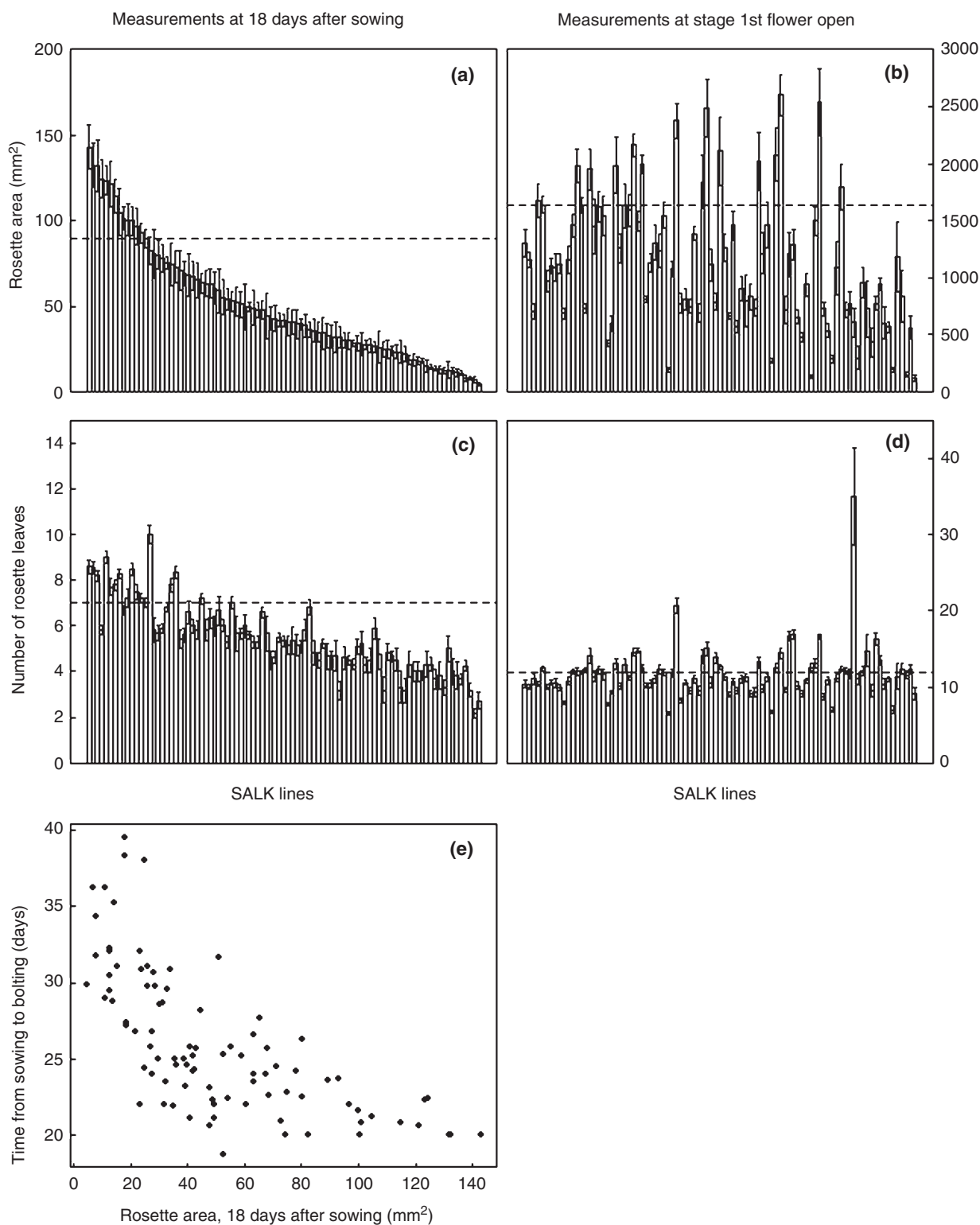
In large collections of natural variants or mutants, hundreds of genotypes have been classified according



**FIGURE 2** | Increasing the throughput of leaf growth phenotypic analyses as illustrated here by the PHENOPSIS platform.<sup>26</sup> In a growth chamber, 504 plants are grown together where they are automatically imaged by top-view and side-view cameras (a, b, c, d). Total rosette area and leaf angles are measured over time on these images. In certain conditions, when leaves do not overlap, individual leaf areas can be measured on top view images (e, inset) and corrected by leaf angles (d). Changes in total rosette area and/or individual leaf area can be plotted over time and sigmoids can be fitted on these curves (e). In addition, at specific dates or stages, different growth traits can be measured at different scales on each plant by destructive measurements as shown for a nonexhaustive list of traits in (f).

to leaf size, shape, or number.<sup>43,45,46,48–50</sup> Many mutants with small rosette areas were identified in these screens, whereas mutants that display larger rosettes than their wild-type are uncommon.<sup>45,22,51</sup> In contrast, a large variability in rosette area is found in populations of recombinant inbred lines, with similar proportions of lines with increased or decreased rosette area compared to the parental lines.<sup>43,50,52,53</sup> Quantitative trait loci (QTL) that control leaf production and/or expansion have been identified when recombinant inbred lines from a same population were grown together in high-throughput screens.<sup>43,44,50</sup> Alleles or allelic combinations at one or a few QTLs that increase leaf area have been described and conclusions on the genetic controls of ‘leaf growth’ or ‘leaf production’ have been drawn.<sup>43,44</sup>

In these studies, leaf growth traits were measured at a given date after sowing or at a given stage. However, several examples illustrate that a static ‘picture’ of a growth trait at a given date or stage does not necessarily reflect what will be the final value of this trait. For example, when a set of 91 genotypes grown together was classified according to total rosette area or number of leaves 18 days after sowing (Figure 3(a) and (b)), the ranking did not reflect what was observed at flowering when rosette development had stopped (Figure 3(c) and (d)). Interestingly, in this dataset, there was a negative correlation between rosette area measured 18 days after sowing and the duration of leaf production estimated as the number of days between the sowing date and bolting (Figure 3(e)). The latter stage is defined as the emergence of the



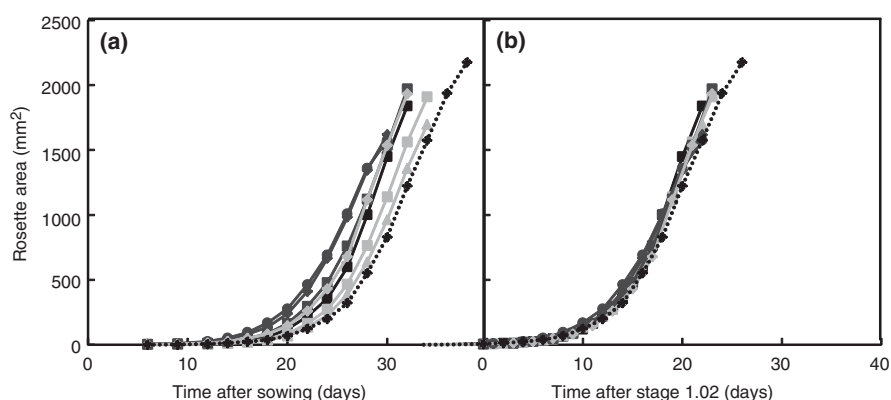
**FIGURE 3** | Leaf growth phenotypes at one date do not necessarily reflect the ‘final’ phenotype. Dissected rosette area and leaf number measured 18 days after sowing are given in (a) and (c), respectively for 90 SALK T-DNA lines and Col-0. The same data at flowering are given in (b) and (d), respectively. Dotted lines correspond to Col-0 values. Data are means with error bars for each genotype ( $n = 7$ ). In all panels, genotypes are ranked according to their increasing rosette area at 18 days. The relationship between the time to bolting and the rosette area at 18 days is shown in (e). Growth conditions and genotypes are described in Supplementary Tables 1 and 2. Methods are given in Supplementary Information 1 and 2.

inflorescence in the center of the rosette, just after the last rosette leaf has emerged.<sup>1</sup> This illustrates that rosettes expanding rapidly early in development have a tendency to stop producing leaves and transit to the reproductive phase earlier than those that expand more slowly in the beginning of their development (Figure 3(e)) and do not necessarily have a larger final leaf area (Figure 3(d)).<sup>22</sup> The tradeoff between the initial rate of growth and growth duration is embedded in the evolutionary framework of life-history theory which predicts that the covarying values of individual characters are constrained by the use of a limited amount of resources (material, time, etc.).<sup>53</sup> In annual plants such as *A. thaliana*, fitness increases with body size, which can be achieved by high relative growth rate or by extending the period of growth.<sup>54,55</sup> Interestingly a similar trade-off has been observed at the individual leaf scale.<sup>15</sup> Genotypes with high initial relative leaf expansion rate have generally a shorter duration of leaf expansion.<sup>15,22</sup> All these examples support that conclusions drawn on traits measured at a given date cannot be generalized to the whole growing period and another analysis at another date or stage may lead to different conclusions, whatever the scale of growth analysis might be.

### PHENOTYPING LEAF GROWTH OVER TIME: LATE DIFFERENCES CAN BE DUE TO EARLY PROCESSES

When individual leaf area or whole rosette area is plotted against time, it produces a sigmoid curve (Figures 1 and 2(e)). In many examples, such as in that in Figure 1, models are fitted to the experimental data set and genotypes are compared on the basis of parameters calculated from the selected model (see

Figure 1 legend). An original logistic model was used as a basis for several extended more generalized models (reviewed in ref 56). One limit to the comparison of leaf growth curves is due to the difficulty of measuring early stages of leaf expansion. Even though recent advances in microscopy and image analysis now allow for the assessment of early stages of leaf growth from leaf initiation on the meristem until emergence, the throughputs of these early measurements are typically low because of the technical constraints associated with destructive measurements and the necessity to grow a high number of plants together for sufficient replicates at each sampling time point.<sup>3,22,57</sup> At the whole plant scale, these methods have shown that subtle differences in shoot apical meristem volumes contribute to subsequent differences in leaf emergence rate and rosette expansion rate.<sup>57,58</sup> Similarly, at the individual leaf scale, early changes in leaf expansion rate or changes in the initial size of the primordium impact on growth dynamics later on and can alter final leaf size.<sup>11</sup> This is due to the exponential behavior of early growth phase in which absolute leaf expansion rate at a given time depends on the leaf area at that time. It is then easy to misinterpret growth curves, as early developmental variation can cause misleading differences in late expansion.<sup>29,31</sup> As an example, at the whole plant scale, differences in rosette area observed at a given date after sowing in a set of genotypes (Figure 4(a)) are no longer apparent when the *x*-scale is expressed in ‘days after leaf 2 emergence’ revealing here that differences in leaf area observed throughout the growing period were due to differences in germination rates and/or early development (Figure 4(b)). This illustrates that very different conclusions can be drawn if the time scale is normalized by developmental stages.<sup>29,31</sup> It is important to keep



**FIGURE 4** | Differences in rosette area at a given time point can be due to differences in early development. Initial dynamics of whole rosette area over time expressed either as days after sowing (a) or days after stage 1.02 (b), i.e., when the second leaf emerged in the center of the rosette.<sup>1</sup> Rosette area was determined on top view images automatically taken in the PHENOPSIS platform (see Figure 2(a)–(c)). Each point is the mean of three plants. Growth conditions and genotypes are described in Supplementary Table 1. Methods are given in Supplementary Information 1 and 2.

in mind that phenotypic differences reported at a given date after sowing can be due to differences in germination rate.

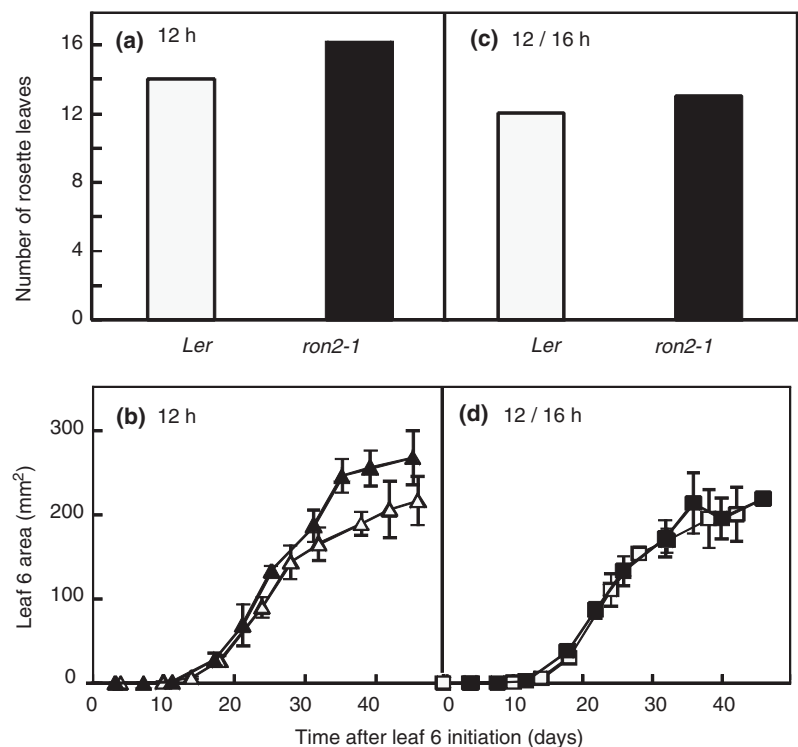
### PHENOTYPING LEAF GROWTH OVER TIME: EARLY DIFFERENCES CAN BE DUE TO LATE PROCESSES

As in other determinate flowering plants, *A. thaliana* shoot development is characterized by distinct phases—a juvenile vegetative phase, an adult vegetative phase during which the plant is reproductively competent, and a reproductive phase.<sup>59,60</sup> In many examples, leaves produced during the same phase share common morphological characteristics such as, circularity, serration, or trichome distribution.<sup>61–63</sup> The number of leaves produced during each phase varies among genotypes. As a consequence, a leaf at a given rank on a rosette with a given leaf number does not have the same ‘phenotypic status’ as a leaf at the same rank on a rosette with more leaves. They will not share the same morphological characteristics cited above. In addition they will also not experience the same microenvironmental conditions that could affect their growth. The sixth leaf on a rosette with a final number of 30 leaves does not experience the same leaf temperature, incident light, and air humidity as the sixth leaf on a rosette with a final number

of six leaves, because an increase in leaf number is associated with an increase in leaf overlapping. Such variability in rosette leaf number has been observed in the *Ler* × *An-1* population of recombinant inbred lines when all 120 lines were grown together in the same environmental condition.<sup>43</sup> Given the important variability in total leaf number among these lines, comparing area and epidermal cell densities in their leaf 6 may not be so pertinent. In addition to the role of shoot architecture on the phenotypic values observed on individual leaf, the development of a leaf at a given rank may or may not have coincided with processes associated to whole plant reproduction. Evidence of tight relationships between vegetative development and floral transition has been recently highlighted in *A. thaliana*.<sup>64</sup> The floral inducer FT has been shown to control—either directly or indirectly—some Squamosa Promoter Binding Protein-like (SPL) genes that promote the juvenile-to-adult transition and exert some control on phylochron, leaf area, and trichome production on leaf abaxial surface.<sup>65–67</sup>

High-throughput phenotypic screens combined with statistical modeling tools have also highlighted these tight relationships, showing that delaying flowering impacts upon individual leaf expansion.<sup>43</sup> In addition, when flowering is delayed by shortening day-length or cutting floral buds and leaf area duration of expansion and epidermal cell area in an individual leaf are increased.<sup>13</sup> It was then suggested

**FIGURE 5** | Differences in final individual leaf area can be due to differences in flowering date: example of *ron2-1* and *Ler* grown with or without synchronization of their flowering dates. Final number of rosette leaves of *Ler* wild-type (light gray) and *ron2-1* (black) plants grown under a constant day length of 12 h (a) or transferred from 12 to 16 h after 15 days (c). Changes with time of leaf 6 area of *Ler* (light gray) and *ron2-1* (black) grown under the photoperiods of 12 h (b) or transferred from 12 to 16 h after 15 days (d). Data are means with 95% intervals of confidence ( $n = 5$  plants per genotype and treatment). Growth conditions and genotypes are described in Supplementary Table 1. Methods are given in Supplementary Information 1 and 2.

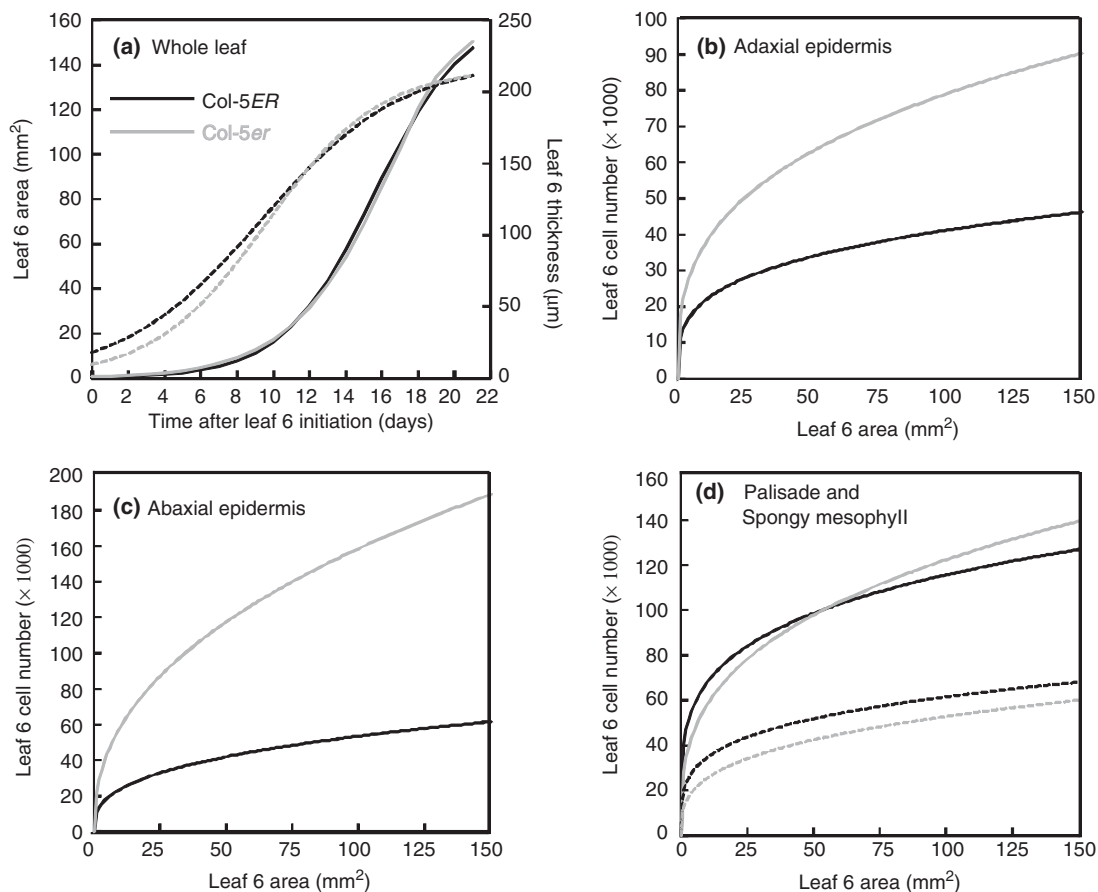


that the leaf growth phenotype observed in late flowering genotypes could be due, at least partly, to the delay in flowering time itself. Comparing the dynamics of leaf production and expansion in a few mutants in the *Ler* background, *ron2-1* was identified as the only one with an increased leaf size compared to its wild-type.<sup>22,68</sup> The final size of leaf 6 was increased in *ron2-1* (Figure 5(a)) as previously observed on the three first leaves.<sup>68</sup> Overall, rosette leaf number was significantly increased in *ron2-1* in comparison with its wild-type (Figure 5(b)). Transferring plants to long day conditions during early development triggered a synchronization of flowering time in both genotypes and resulted in similar phenotypes in terms of leaf number and individual leaf area (Figure 5(c) and (d)). Thus, even though there is no doubt that *ron2* mutation did affect individual leaf growth, it seemed to be an indirect effect primarily due to an effect on

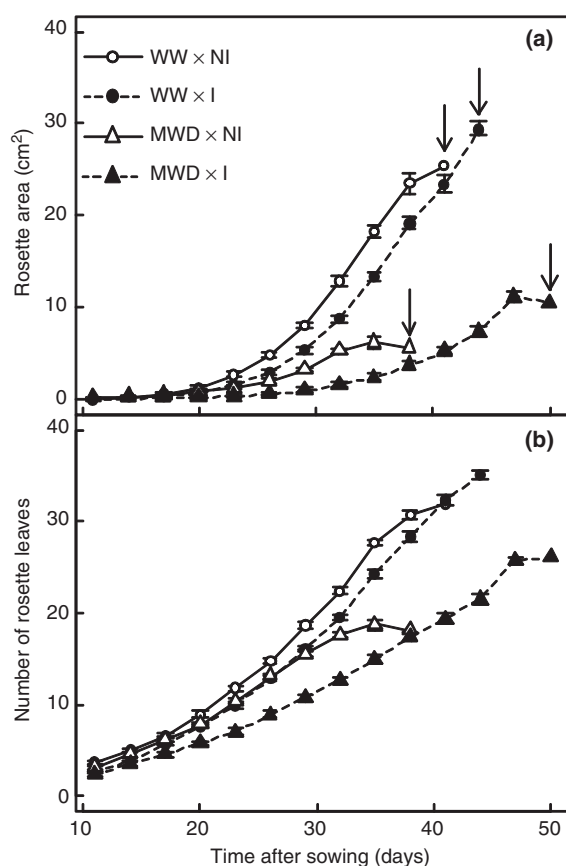
leaf production and/or floral transition. There is then a real difficulty in comparing leaf growth phenotypes between genotypes exhibiting different flowering date and number of leaves. There is no means to take this into account at the moment and it is a real challenge to develop mathematical formalisms to fill this gap in leaf growth phenotyping.

### THE SAME FINAL LEAF SIZE CAN BE REACHED VIA DIFFERENT LEAF STRUCTURES: INCIDENCE ON CELLULAR PHENOTYPING

Techniques and frameworks of analyses have been developed to quantitatively and dynamically assess the cellular processes underlying shoot organ formation and growth.<sup>14,69</sup> Using these methods, cellular



**FIGURE 6** | Phenotyping cell density in a given tissue does not necessarily reflect the cellular phenotype of other tissues: example of the effects of the *erecta* mutation on cell number in different leaf tissues. Changes over time in leaf surface area (full lines) and thickness (dotted lines) (a), for the sixth leaf of *Arabidopsis thaliana* Col-5 wild-type (Col-5ER, black) and Col-5 harbouring the *erecta* mutation (Col-5er, gray). Cell number increases during leaf blade expansion are shown for the adaxial epidermis (b) and the abaxial epidermis (c), revealing a strong effect of the *y* mutation on the dynamics of cell division in these tissues. Cell number increases during leaf blade expansion are also shown in the palisade (d, solid lines) and spongy mesophyll (d, dotted lines), revealing similar dynamics of cell number changes between the two genotypes in these two tissues. Growth conditions and genotypes are described in Supplementary Table 1. Methods are given in Supplementary Information 1 and 2.



**FIGURE 7** | Responses of rosette area (a) and leaf number (b) of *Arabidopsis* Col-0 to water deficit and bacterial inoculation at one date do not reflect the final responses. Plants were grown under four soil conditions: well watered (WW) and moderate water deficit (MWD), with (I) or without (NI) soil inoculation with a plant growth promoting rhizobacteria (PGPR), *Phyllobacterium brassicacearum*.<sup>83,84</sup> In both watering conditions, bacterial inoculation has a negative effect on leaf number and rosette area during a first part of plant development. For instance, rosettes of inoculated plants are smaller and have a lower number of leaves than noninoculated plants 30 days after sowing. However, at the end of the vegetative period (i.e., bolting stage shown by arrows in (a)), the rhizobacteria has a significant promoting effect on both leaf number and rosette area. This promoting effect is more pronounced in the MWD condition than in the WW treatment. Inoculated plants have lower leaf emergence and rosette expansion rates from germination to the emergence of flower buds, but they delay flowering and finally produce a higher number of leaves and larger rosette areas than noninoculated plants (a, b). Error bars are standard errors of mean values ( $n = 11-13$ ). Growth conditions and genotypes are described in Supplementary Table 1. Methods are given in Supplementary Information 1 and 2.

behaviors underlying leaf shape and size variations have been assessed in large collection of mutants, populations of recombinant inbred lines, and naturally occurring accessions, giving insights into the genetic control of these variables as well as their coordination and role during leaf development.<sup>43,45,46,70,71</sup> In most

cases, cell density, cell area, and cell number are determined on epidermal peels, imprints, or paradermal views of cleared leaves. These measurements are generally limited to the upper epidermis mainly because of technical constraints, but also because the epidermis is often considered as the tissue physically limiting whole leaf expansion.<sup>72</sup> Indeed, mutants with strong changes in mesophyll cell density, but no effect on leaf size, have been described, whereas epidermal cell size or number is often positively correlated with leaf area.<sup>43,45,73</sup> Some genotypes do however present strong changes in epidermal cell number and size without exhibiting any change in leaf area.<sup>46,74</sup> For example, a drastic modification in epidermal cell density is caused by the *erecta* mutation without any effect on leaf size (Figure 6(a)–(c)). Cell densities, together with stomatal densities, are higher for both the adaxial (2×) and abaxial epidermis (3×) from very early on in leaf development (5 days after initiation), while intriguingly, these changes in the epidermis are not reflected in internal layers as cell number is not affected in both the spongy and palisade mesophyll (Figure 6(d)). In the mesophyll, cell division stops 9 days after leaf initiation and is then followed by a phase of cell expansion only, whereas cell division in epidermal tissues extends into the phase of maximum surface area expansion of the whole leaf: as the leaf area grows, the epidermal tissues enlarge by a combination of cell division and expansion. In this example, the stability of final leaf size is thus associated with stability in mesophyll cell densities while epidermal development is highly disrupted. As a consequence, epidermal cellular variables cannot be used as a proxy of leaf size. Recent studies have demonstrated that, in addition to stomata and leaf surface, mesophyll conductance plays a major role in the rate of carbon fixation, the primary determinant of biomass accumulation.<sup>75</sup> Therefore, the use of high-throughput 2D-imaging to characterize leaf growth misses its 3D-structure, although the latter is an important source of variation in leaf functioning. This limitation can be partly overcome by the estimation of the specific leaf area (SLA), which is defined by the ratio ‘leaf surface:leaf dry mass’ and is associated with the variation in leaf density and leaf thickness. Numerous studies in the field of ecology and, more recently, on *A. thaliana*, have reported tight links between SLA and the rate of net photosynthesis.<sup>53,76,77</sup> However, the measurement of leaf dry mass is destructive and generally not compatible with high-throughput experiments. Therefore, there is a real challenge to develop new methods to automatically and repeatedly estimate 3D growth of leaves in large populations.



## CONCLUSION

Frameworks of analyses were developed mainly during the 1980s to analyze quantitatively shoot growth from cell to whole organ with precise spatial and temporal resolutions.<sup>78–82</sup> These studies laid the ground work of what is presented here and are largely neglected today. Because of rapid advances in plant genomics, many groups have developed automated phenotyping platforms to try to bridge the genotype to phenotype gap. It has long been known that different genotypes have different rates of shoot development and different spatial distributions of growth in organs and tissues but, the development of phenotyping platforms has led to a simplification of these frameworks of analysis and as such important considerations are often forgotten. Simplified screens can lead to draw distorted conclusions or to reduce or miss the subtlety of some phenotypes. Examples presented here may guide future phenotypic campaigns or help to interpret existing or future datasets with objectivity. Illustrations were based on comparing *A. thaliana* leaf growth and development between genotypes,

but conclusions are not restricted to this species, to this organ, or to this type of comparisons. The same precautions have to be taken when analyzing growth responses to environmental conditions. For example, evidence of a trade-off between a lower leaf growth rate and a longer leaf growth period has been reported in response to drought or day-length<sup>13,26</sup> and can also be found in response to biotic factor (Figure 7). The increase in shoot development usually reported by soil inoculation of a plant growth promoting rhizobacteria<sup>83,84</sup> is not visible early during leaf development, but it appears later on as the consequence of a lengthened period of both leaf production and whole rosette development (Figure 7).

In all phenotypic studies, a good knowledge of the studied system as well as mathematical and modeling tools are necessary to interpret phenotypic datasets. This does not question or cast doubt on all phenotypic datasets that have been published without taking into account the considerations presented here. However, this could help to mitigate some interpretations of growth phenotypes or guide future research when growth is compared among different genotypes or environmental conditions.

## ACKNOWLEDGMENTS

This review was made possible by grants from the AGRON-OMICS integrated project funded in the sixth European Framework Programme (LSHG-CT-2006-037704) and from the Agropolis Fondation Montpellier, France (RTRA project no 07047) that provided grants for C.M. and N.W., respectively. F.V. and M.L. were funded by CIFRE grants (ANRT, French Ministry of Research) supported by BAYER Crop Science (contracts 0398/2009-09 42 008 and 713/2011-11 42 010, respectively). S.T. was supported by a grant from GENOPLANTE (GPLA-06014G). S.C. was funded by the European Community Human Potential Program (HPRN-CT-2002-00267) as part of the DAGOLIGN Research Training Network. J.B. was funded by the French Ministry of Higher Education and Research. The PHENOPSIS platform presented in Figure 2 is part of the European Plant Phenotyping Network (EPPN, grant agreement no. 284443) funded by the FP7 Research Infrastructures Programme of the European Union.

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## Supporting information

### SI. 1 Micrometeorological conditions.

In all six experiments (Figures 1, 3, 4, 5, 6 and 7) seeds of the different genotypes (Supplementary Table 1) were sown in pots filled with a mixture (1:1) of a loamy soil and organic compost. Just after sowing pots were placed in a growth-chamber under controlled air temperature, air humidity and incident light. Micrometeorological conditions were kept constant during the whole duration of the experiments for experiments 1, 2, 3, 4, 5 and 6. In Experiment 4, half of the plants were grown at a 12h day length during whole experiment whereas the other half was transferred from 12 to 16 h day length at 15 days after sowing. Light intensity in the 16h condition was reduced with filters to reach a similar daily incident PPFD than in the 12h condition. Mean values of each micrometeorological condition are given in Supplementary Table 1. Each pot was watered daily with a modified one-tenth strength Hoagland's solution.<sup>S1</sup> Experiments 2, 3, 5 and 6 were performed in a growth chamber equipped with the PHENOPSIS automaton (Figure 2 (a), (b)). During Experiment 7, plants were grown under four treatments combining two watering regimes with (I) or without (NI) soil inoculation by a plant growth promoting rhizobacteria *Phyllobacterium brassicacearum*, strain STM196. Depending on the watering regime, soil water content was adjusted daily to either 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil (well-watered condition, WW) or 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil (water deficit condition, WD). The inoculum of STM196 was adjusted to obtain 3.10<sup>7</sup> colony forming units per gram of soil.

**Table S1 Genotypes and mean environmental conditions during experiments 1 to 6.**

	Genotypes	Air temp. (°C)	Relative air humidity (%)	Instantaneous incident PPFD ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	Day length (h)
Exp 1 (Fig 1)	Landsberg <i>erecta</i> ( <i>Ler</i> ) ecotype <i>elo-1</i> ( <i>Ler</i> background)	22.9	74	260	10
Exp 2 (Fig 3)	Columbia-0 ( <i>Col-0</i> ) ecotype 90 T-DNA insertions lines ( <i>Col-0</i> background) <sup>†</sup>	21.5	63	168	16
Exp 3 (Fig 4)	Columbia-0 ( <i>Col-0</i> ) ecotype 7 epigenetic recombinant lines (epiRILs)*	21	69	180	16
Exp 4 (Fig 5)	Landsberg <i>erecta</i> ( <i>Ler</i> ) ecotype <i>ron2-1</i> ( <i>Ler</i> background)	19.3	73	279 279 → 205	12 12 → 16
Exp 5 (Fig 6)	Columbia-5 <i>ER</i> ( <i>Col-0</i> with <i>gl1-1</i> marker) Columbia-5 <i>er</i>	20.5	72	166	16
Exp 6 (Fig 7)	Columbia-0 ( <i>Col-0</i> ) ecotype	19.5	67	185	12

<sup>†</sup>List of the lines is given in Supplementary Table 2. \*Lines are described in Ref S2.

## SI. 2 Phenotyping methods in each experiment.

**Experiment 1 (Figure 1).** Areas of leaves at position 6 on the rosette were measured at intervals of 2 to 3 days from initiation to the end of expansion of the leaf. From leaf initiation to leaf emergence, this was done by dissecting the apex of 5 plants in a drop of water under the microscope. The area of the excised leaf 6 was measured with image analysis software (Bioscan-Optimas V 4.10, Edmonds, WA). After leaf emergence, leaf area of 6 plants was measured with the aforementioned image analysis software on digital photographs until the end of leaf expansion. A sigmoidal curve was fitted to the curve relating leaf expansion to time:

$$y = \frac{A}{1 + e^{-[(t-X_0)/B]}} \quad (\text{Eq. 1})$$

Absolute leaf expansion rate at time  $j$  ( $LER_j$ ) was calculated from initiation to the end of expansion as the local slope (at time  $j$ ) of the relationship between leaf area ( $LA$ ) and time: <sup>11</sup>

$$LER_j = [d(LA)/dt]_j \quad (\text{Eq. 2})$$

Relative leaf expansion rate at time  $j$  ( $RER_j$ ) was calculated from initiation to the end of expansion as the local slope (at time  $j$ ) of the relationship between the logarithm of leaf area ( $LA$ ) and time: <sup>11</sup>

$$RER_j = [d(\ln LA)/dt]_j \quad (\text{Eq. 3})$$

**Experiment 2 (Figure 3).** Fourteen plants of each line were grown together. Seven plants were harvested 18 days after sowing whereas the others were harvested at a common stage of plant development, stage 6.00, i.e. first flower open (as defined in Ref 1). The successive leaves of the rosette were excised without their petiole, stuck on a sheet of paper and scanned as shown in Figure 2 (f). Individual leaf areas were measured with image analysis software (ImageJ 1.43C,

Wayne Rasband, National Institutes of Health, USA).<sup>S3</sup> Rosette area was calculated as the sum of individual leaf areas. Rosette leaves were counted after rosette dissection. The time from sowing to bolting was determined as the time elapsed between the sowing date and the emergence of the inflorescence, i.e. the bolting stage. This stage was determined by naked-eye, on daily zenithal images taken by the PHENOPSIS automaton (Figure 2 (c)).

[Lines are kind gifts from the group of José Luis Micol, Universidad Miguel Hernandez, Elche, Alicante, Spain].

**Experiment 3 (Figure 4).** Projected area of the rosette was determined every 3 days from semi-automated analysis (ImageJ 1.43C) of zenithal images of the plants taken by the PHENOPSIS automaton from germination to first silique formation (Figure 2 (c)).

[Lines are kind gifts from Jerzy Paszkowski, University of Geneva, Geneva, Switzerland].

**Experiment 4 (Figure 5).** Leaf 6 area was measured as described in the method section of Experiment 1. Rosette leaves were counted at the end of plant growth.

[Genotypes are kind gifts from Mieke Van Lijsebettens, Department of Plant Systems Biology, Flanders Institute for Biotechnology, Gent, Belgium].

**Experiment 5 (Figure 6).** Leaf 6 area was measured as described in the method section of Experiment 1, from stage 1.02 (two visible leaves) to 6.00 (first flower open) (as defined in Ref 1). Six plants per time point were harvested for the measurement of leaf 6 thickness and cellular growth variables by three-dimensional imaging. Whole seedlings or leaves were fixed, conserved and subsequently cleared and stained using propidium iodide as described in Ref 3. Image stacks covering the complete leaf thickness were produced for the middle of the leaf along the



longitudinal axis, and approximately midway between the leaf midvein and margin using multiphoton laser scanning microscope. The quantitative analysis of tissue and cell dimensions in image stacks was performed by means of specifically developed ImageJ macros and R scripts (Ref 3; R Development Core Team, 2010).<sup>S3-4</sup> Kinematic and statistical analyses were performed in R. A sigmoid function was fitted to leaf surface area and thickness expansion profiles over time, defined as the number of days after the initiation of the 6th leaf, to extract kinematic growth variables (Eq. 1). Power plots were fitted to leaf cell number data versus expansion in surface area for the adaxial and abaxial epidermis and the palisade and spongy mesophyll.

**Experiment 6 (Figure 7).** Projected area of the rosette was determined every 3 days from semi-automated analysis (ImageJ 1.43C) of zenithal images of the plants taken by the PHENOPSIS automaton from germination to bolting (Figure 2 (c)). Bolting was determined by macroscopic visualization of flower buds (stage 5.01, Ref 1). For each plant, the number of visible leaves was counted every 2 to 3 days by naked-eyes.

[The *Phyllobacterium brassicaceraum* bacteria (STM196) was provided by Bruno Touraine and Fabrice Varoquaux, Université Montpellier II, Montpellier, France]

**Table S2 List of the T-DNA insertion lines grown in Experiment 2 (Figure 3).**

Ninety T-DNA insertion lines were selected for their contrasted leaf growth phenotypes identified in a previous screen performed by the group of José Luis Micol (Universidad Miguel Hernandez, Elche, Alicante, Spain). In this table, lines are ranked from the one with the highest mean rosette area to the one with the lowest mean rosette area 18 days after sowing. The dotted line indicates the rank of the Col-0 accession (wild-type).

SALK Line	Gene	SALK Line	Gene	SALK Line	Gene
SALK_138229C	At1g54840	SALK_001496C	At1g62430	SALK_048175C	At1g30450
SALK_080604C	At1g08135	SALK_007854C	At4g11120	SALK_151603C	At3g62980
SALK_119457C	At4g30410	SALK_015522C	At5g36880	SALK_145086C	At1g13740
SALK_018664C	At4g09000	SALK_001004C	At2g30810	SALK_075661C	At3g13228
SALK_032963C	At5g16270	SALK_034684C	At2g22420	SALK_130499C	At4g31390
SALK_085503C	At2g33420	SALK_020615C	At1g76470	SALK_065118C	At1g53140
SALK_143422C	At2g44650	SALK_024759C	At1g48950	SALK_063595C	At1g20640
SALK_054681C	At5g46115	SALK_035676C	At1g10050	SALK_057785C	At1g22090
SALK_009736C	At1g17130	SALK_056529C	At1g20860	SALK_045025C	At3g20550
SALK_045623C	At5g65050	SALK_022117C	At2g01290	SALK_079285C	At3g17040
SALK_021217C	At3g47640	SALK_067582C	At2g32540	SALK_136507C	At1g78020
SALK_145203C	At4g08540	SALK_148403C	At5g61950	SALK_129037C	At4g13590
SALK_026667C	At5g28250	SALK_048174C	At4g11540		
SALK_126071C	At1g04730	SALK_057052C	At5g54770		
SALK_053198C	At5g24470	SALK_072771C	At2g07540		
SALK_019994C	At1g55370	SALK_061494C	At5g35220		
SALK_019359C	At4g10920	SALK_017692C	At2g29670		
SALK_025598C	At2g46970	SALK_142112C	At1g74940		
SALK_080188C	At5g58970	SALK_009798C	At2g23090		
SALK_003718C	At3g16950	SALK_008561C	At4g20290		
SALK_011867C	At5g51750	SALK_040660C	At1g79090		
SALK_030786C	At3g46790	SALK_086630C	At3g50400		
SALK_011586C	At3g27620	SALK_101771C	At1g18500		
SALK_041291C	At2g22680	SALK_003711C	At1g71760		
SALK_033455C	At3g28860	SALK_045034C	At5g17660		
SALK_129352C	At3g30180	SALK_067017C	At5g52440		
SALK_145983C	At4g09340	SALK_019175C	At2g31725		
SALK_066708C	At4g24175	SALK_012771C	At4g25410		
SALK_075797C	At5g51770	SALK_005153C	At5g13680		
SALK_044119C	At2g35040	SALK_144264C	At3g19510		
SALK_037549C	At2g23220	SALK_070464C	At1g74420		
SALK_004741C	At1g42980	SALK_014243C	At3g52105		
SALK_125189C	At1g61310	SALK_122867C	At1g18900		
SALK_117972C	At2g44100	SALK_015088C	At5g61960		
SALK_021759C	At4g32200	SALK_021618C	At2g47490		
SALK_034227C	At5g59950	SALK_046141C	At2g03190		
SALK_055996C	At1g32500	SALK_064915C	At5g23980		
SALK_020801C	At2g01450	SALK_049200C	At2g04043		
SALK_148633C	At4g20450	SALK_055458C	At1g14040		

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## ***Chapitre 3***

# ***Plant-bacteria interaction under moderate water stress***

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# The PGPR strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delay and physiological changes that result in improved drought tolerance in *Arabidopsis*

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Received: 25 April 2013

Accepted: 24 May 2013

New Phytologist (2013)

doi: 10.1111/nph.12383

**Key words:** *Arabidopsis thaliana*, drought tolerance, flowering phenology, *Phyllobacterium brassicacearum* STM196, plant growth-promoting rhizobacteria (PGPR), water-use efficiency.

## Summary

- Understanding how biotic interactions can improve plant tolerance to drought is a challenging prospect for agronomy and ecology. Plant growth-promoting rhizobacteria (PGPR) are promising candidates but the phenotypic changes induced by PGPR under drought remain to be elucidated.
- We investigated the effects of *Phyllobacterium brassicacearum* STM196 strain, a PGPR isolated from the rhizosphere of oilseed rape, on two accessions of *Arabidopsis thaliana* with contrasting flowering time. We measured multiple morphophysiological traits related to plant growth and development in order to quantify the added value of the bacteria to drought-response strategies of *Arabidopsis* in soil conditions.
- A delay in reproductive development induced by the bacteria resulted in a gain of biomass that was independent of the accession and the watering regime. Coordinated changes in transpiration, ABA content, photosynthesis and development resulted in higher water-use efficiency and a better tolerance to drought of inoculated plants.
- Our findings give new insights into the ecophysiological bases by which PGPR can confer stress tolerance to plants. Rhizobacteria-induced delay in flowering time could represent a valuable strategy for increasing biomass yield, whereas rhizobacteria-induced improvement of water use is of particular interest in multiple scenarios of water availability.

## Introduction

Drought is one of the major limitations to food production worldwide. The development of drought-resistant cultivars and water-use-efficient plants is therefore of global concern. In their habitats, plants are not single organisms but are surrounded by dense populations of diverse microorganisms with which they probably interact. Some of these plant-microorganism interactions are beneficial for plant growth and allow plants to better cope with biotic and abiotic stresses (Yang *et al.*, 2009).

Drought periods lead to large physiological and developmental alterations in plants. Water deprivation decreases above-ground vegetative biomass accumulation and therefore reduces plant performance (Boyer & Westgate, 2004; Hummel *et al.*, 2010; Tardieu *et al.*, 2011; Vile *et al.*, 2012). Vegetative growth and the total production of dry matter are closely related to key developmental switches such as reproductive transition (Jung & Muller, 2009). Specifically, flowering time can be delayed (McMaster *et al.*, 2009; Tisne *et al.*, 2010) or hastened (Verslues & Juenger, 2011) in response to drought, most likely depending upon plant species and the occurrence, duration and severity of the stress.

In order to minimize the negative effects of water deficit and complete their life cycle under unfavourable conditions, plants exhibit a variety of strategies (for reviews, see Farooq *et al.*, 2009; Verslues & Juenger, 2011). Physiological changes occur rapidly after the onset of water deficit in order to maintain high tissue water potential. One of the swiftest responses is a reduction of transpiration through reduced leaf conductance following stomata closure. This response is often associated with an accumulation of ABA or enhancement of sensitivity to this hormone in the leaf cells, leading to the induction of related signalling genes (Harb *et al.*, 2010). Rapid osmotic adjustment through active accumulation of solutes also helps in maintaining cell turgor and increases the driving force of water influx into the cell (Yoshida *et al.*, 1997). In the long term, increased root-to-shoot ratio, through reduced above-ground growth and/or increased root growth, participates in reducing evaporative area and increasing water absorption capacities from the soil (Boyer, 1985), together contributing to increased water-use efficiency (WUE). WUE reflects the tradeoff between CO<sub>2</sub> acquisition for growth and water losses and is therefore an important indicator of how plants manage water stress (Blum, 2005; Tardieu, 2012).

Soil microorganisms may interact with plant-specific mechanisms related to drought resistance. Some naturally occurring free-living soil bacteria, namely plant growth-promoting rhizobacteria (PGPR), colonize the root system and maintain mutualistic interactions that lead to plant growth improvement and plant protection against multiple stresses, including drought, salt, heavy metals or pathogens (Dimkpa *et al.*, 2009; Lugtenberg & Kamilova, 2009; Yang *et al.*, 2009). PGPR such as *Azospirillum*, *Azotobacter* and *Pseudomonas fluorescens* are well known for their plant growth-promoting effects and are notably used for improving crop yields (Kloepper *et al.*, 1989; Lucy *et al.*, 2004). PGPR effects involve multiple changes in plant metabolism and signalling networks (Lugtenberg & Kamilova, 2009; Friesen *et al.*, 2011). Modifications in phytohormone content and/or signalling have been reported (see, for review, Dodd *et al.*, 2010), such as decreased ethylene production via bacterial ACC deaminase activity (Glick *et al.*, 1998; Belimov *et al.*, 2009), changes in cytokinin-ABA balance (Figueiredo *et al.*, 2008; Cohen *et al.*, 2009) or changes in auxin signalling (Persello-Cartieaux *et al.*, 2003; Contesto *et al.*, 2010). These effects on hormone pathways are likely to interfere with plant tolerance to drought stress. Some PGPR strains improve plant enzyme activity, such as catalase or superoxide dismutase, which alleviates the oxidative damage induced by drought (Kohler *et al.*, 2008; Wang *et al.*, 2012). Finally, PGPR have been shown to increase drought-response transcript abundances (Wang *et al.*, 2005, 2012).

Despite strong evidence that PGPR influence overall plant performance, their detailed effects on development, growth and physiology under drought have been less well explored. Therefore, integrative studies to explain how PGPR can improve drought tolerance are lacking. Among the specific PGPR-mediated mechanisms identified is the enhancement of wheat growth by *Azospirillum* sp. strains under various drought intensities, which was associated with better maintenance of plant water status as a result of increased cell wall elasticity (Creus *et al.*, 2004). An increase of photosynthetic capacity has also been shown in *Pinus halepensis* inoculated with *P. fluorescens* (Rincon *et al.*, 2008) or in *Azospirillum*-inoculated rice (Ruiz-Sanchez *et al.*, 2011). Although these physiological studies have detailed measurements of plant water relations, most failed to report drought effect on the dynamics of plant development. Moreover, most studies focus on a single time point, generally at flowering or seed maturity, and reports on plant growth throughout the whole plant cycle are very scarce.

Here, we investigated the growth and physiological responses of *Arabidopsis thaliana* inoculated with a free-living PGPR, *Phyllobacterium brassicacearum* strain STM196 under long-term water deficit. *A. thaliana* is a useful organism to study plant interactions with PGPR (Ryu *et al.*, 2005; Desbrosses *et al.*, 2009), but the effects of PGPR on the development or physiology of this model species under water stress have been little investigated. STM196 belongs to the *Phyllobacteriaceae* family in the *Rhizobiales* order of  $\alpha$ -*Proteobacteria* (Mantelin *et al.*, 2006). This strain was the most efficient PGPR isolated from the rhizoplane of field-grown *Brassica napus* roots (Bertrand *et al.*, 2001; Larcher *et al.*, 2003).

Previous *in vitro* studies showed that STM196 enhances shoot and root growth of *A. thaliana*, and modifies its root architecture and hormonal signalling (Mantelin *et al.*, 2006; Contesto *et al.*, 2010; Galland *et al.*, 2012; Kechid *et al.*, 2013). However, we lack information on plant-bacteria interactions under soil conditions and no study has investigated the effects of this particular strain on plant response to drought. We used the high-throughput plant phenotyping platform PHENOPSIS (Granier *et al.*, 2006) to decipher the effects of STM196 on multiple plant traits related to growth dynamics, development and physiology under well-defined soil water availability. First, we show that the plant growth-promoting effect of STM196 is related to a delay in reproductive transition in two *A. thaliana* accessions with contrasting flowering phenology. Then, we show that STM196 induces a suite of physiological and developmental changes that lead to enhanced WUE and to a better plant tolerance to water deficit.

## Materials and Methods

### Bacterial inoculum and soil inoculation

The strain *Phyllobacterium brassicacearum* STM196 was grown for 3 d in Petri dishes on a sterile (20 min at 120°C) 1.5% agar (w/v; Sigma-Aldrich) medium (E') containing 2.87 mM K<sub>2</sub>HPO<sub>4</sub>, 0.81 mM MgSO<sub>4</sub>, 1.71 mM NaCl, 7.91 mM KNO<sub>3</sub>, 0.34 mM CaCl<sub>2</sub>, 30  $\mu$ M FeCl<sub>3</sub>, 1% mannitol (w/v) and 0.3% yeast extract (w/v; Sigma-Aldrich), adjusted to pH 6.8. Next, the bacteria were grown aerobically in 750 ml liquid E' medium on a rotary shaker (145 rpm) at 25°C for 24 h to reach the exponential phase of growth. The culture of bacteria cells was pelleted by centrifugation (3200 g, 15 min, 20°C) and resuspended in water. To obtain 3  $\times$  10<sup>7</sup> colony-forming units (CFU) g<sup>-1</sup> of soil, the volume was adjusted based upon a correspondence with the absorbance measured at 595 nm (WPA UV 1101; Biotech Photometer, Cambridge, UK). This inoculum was placed directly into the soil substrate, which was then manually homogenized.

### Plant material, growth conditions and irrigation treatments

We selected two accessions of *A. thaliana* (L.) Heynh differing in flowering time: Col-0, one of the reference accessions in *A. thaliana* research; and An-1, an early-flowering accession (Granier *et al.*, 2006; Tisne *et al.*, 2010). A total of 96 individual plants per genotype were studied (see Supporting Information, Table S1, for details on replicate numbers per trait and conditions). Five seeds were sown at the soil surface in 260 ml culture pots filled with a damped mixture (1 : 1, v/v) of loamy soil and organic compost (Neuhaus N2) inoculated (or not) with STM196. Noninoculated soil was previously damped with deionized water to avoid difference in initial soil humidity between this soil and inoculated soil. Soil water content was controlled before sowing to estimate the initial amount of dry soil and water in each pot. The 192 pots were placed in the dark at 4°C for 48 h to ensure stratification and were then transferred into the PHENOPSIS growth chamber (Granier *et al.*, 2006). Pots were kept in

the dark for 2 d and were dampened with sprayed deionized water three times a day until germination. Then, plants were cultivated under conditions of 12 h day length ( $180 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD) at plant height). During the germination phase (7 d), the air temperature was set to 20°C day and night, and the relative humidity of the air was adjusted to maintain a constant water vapour pressure deficit (VPD) at 0.6 kPa (Fig. S1). Plants were then grown at 20 : 17°C, day : night temperatures and a VPD of 0.8 kPa (Fig. S1). Soil relative water content was maintained at  $0.35 \text{ g H}_2\text{O g}^{-1}$  dry soil (corresponding to  $-0.07 \text{ MPa}$  soil water potential; WP4-T dewpoint meter; Decagon Devices, Pullman, WA, USA) until the emergence of the first two true leaves (stage 1.02 in Boyes *et al.*, 2001). After stage 1.02, seedlings were thinned to one plant per pot and soil water deficit was started. Soil water content was maintained at  $0.35 \text{ g H}_2\text{O g}^{-1}$  dry soil in the well-watered treatment (WW) and was decreased to  $0.20 \text{ g H}_2\text{O g}^{-1}$  dry soil (corresponding to  $-0.28 \text{ MPa}$  soil water potential) by stopping irrigation (Fig. S2) in the water-deficit treatment (WD). The weight of each pot was adjusted daily with a modified 1/10th-strength Hoagland solution (Hoagland & Arnon, 1950) to reach the target soil water content. These two values of soil water content were maintained until harvest.

#### Measurement of plant traits

**Rosette expansion, leaf production dynamics and phenology** Individual areas of the two first leaves and projected area of the rosette ( $\text{RA}_{\text{proj}}$ ) were determined every 3 d from semi-automated analysis (ImageJ 1.43C; Rasband, Bethesda, MD, USA) of zenithal images of the plants (PROSILICA AVT GC 1600C camera, ALLIED, Stradroda, Germany). The initial relative expansion rate of the rosette ( $\text{RER}$ ,  $\text{d}^{-1}$ ) was estimated as the slope of the linear relationship between total leaf area and time after sowing. A sigmoid curve was fitted for each plant following  $\text{RA}_{\text{proj}} = a/(1 + \exp(-((d-a)/b)))$ , where  $a$  is the maximum area, and  $d$  is the number of days after sowing. The maximum rate of leaf expansion ( $R_{\text{max}}$ ,  $\text{mm}^2 \text{d}^{-1}$ ) was calculated from the first derivative of this logistic model at  $d_0$  as  $R_{\text{max}} = a/(4b)$ . The duration ( $d$ ) of rosette expansion was estimated as the time period for rosette area to increase from 5 to 95% maximum area following  $a/2 - b \log_e((1/0.95) - 1)$ .

The number of leaves that were visible to the naked eye was counted every 2–3 d to determine the phyllochron ( $d$ ), that is, the time necessary to have a new visible leaf, until emergence of the flowering stem. Bolting and flowering time were determined as the number of d from germination until macroscopic visualization of flower buds (stage 5.01; Boyes *et al.*, 2001) and the first flower open (stage 6.00), respectively.

**Whole-plant and leaf morphology** Col-0 individuals were harvested at bolting, and An-1 individuals were harvested both at bolting and at first flower open. Rosettes were cut and immediately weighed after the removal of inflorescence stems to determine above-ground vegetative fresh mass (FM). The rosettes were wrapped in moist paper and placed into Petri dishes at 4°C

in darkness overnight to achieve complete rehydration. Water-saturated fresh mass (SM) was then determined. The total leaf number was determined, and the leaf blades were separated from their petiole and scanned for measurements of leaf area, length and width (ImageJ 1.43C). Leaf blades, petioles and reproductive structures were then oven-dried separately at 65°C for 48 h, and their dry mass (DM) was determined. Rosette DM was calculated as the sum of blade DM and petiole DM. From these measurements, leaf dry matter content ( $\text{LDMC} = \text{DM}/\text{SM}$  ( $\text{mg g}^{-1}$ )) and relative water content ( $\text{RWC} = (\text{FM} - \text{DM}) \times 100 \times (\text{SM} - \text{DM})^{-1}$ ) were calculated at the rosette level. Leaf dry mass per area ( $\text{LMA}$ ,  $\text{g m}^{-2}$ ) was calculated as DM divided by the projected rosette area as determined from the last zenithal image before harvest. Roots were carefully extracted from the soil, gently washed in deionized water, placed in a paper bag at 65°C for 5 d and their dry mass ( $\text{DM}_{\text{root}}$ ) determined.

**Leaf and shoot development** Postembryonic development of the shoot is characterized by distinct phases: a reproductively incompetent juvenile vegetative phase, a reproductively competent adult phase and a reproductive phase (Willmann & Poethig, 2011). In Arabidopsis, during each phase different types of leaves are produced: juvenile, adult and cauline leaves, which can be distinguished from each other by morphological characteristics (Steynen *et al.*, 2001; Willmann & Poethig, 2011). Juvenile leaves are flat and round with a small blade and a long petiole. Generally, juvenile leaves consist of the first two leaves. Adult leaves are recognized by a larger, curled blade and a lanceolate shape, whereas cauline leaves are recognized by their small and pointed leaf blade and lack of petiole (Steynen *et al.*, 2001). We used the leaf blade length-to-width ratio to quantify leaf shape and estimate leaf types. The length of the blade was determined as the distance from the blade-to-petiole junction to the distal leaf tip and the width was determined at the midpoint of this line. This ratio approximates to 1 (rounded leaves) in juvenile leaves and increases in adult leaves (Willmann & Poethig, 2011).

**Stomata and cell density** Adaxial epidermal imprints of the sixth leaf were obtained by drying off a varnish coat spread on the surface of the leaf. The imprint was peeled off and then stuck on microscope slides with one-sided adhesive. Imprints were placed under a microscope (Leitz DM RB; Leica, Wetzlar, Germany) coupled to an image analyser (BioScan-Optimas 4.10, Edmond, WA, USA). Mean cell density and stomatal density were then determined in two  $0.12 \text{ mm}^2$  zones in the middle part of the leaf blade distributed on both sides of the midvein, halfway from the margins distributed on both sides. The stomatal index was calculated as  $100 \times \text{stomatal number}/(\text{stomatal number} + \text{cell number})$ .

**Net photosynthetic and transpiration rates** Gas exchanges were determined at the bolting stage, that is, just before harvest, only in the Col-0 accession. The rate of  $\text{CO}_2$  assimilation was measured using a whole-plant chamber designed for Arabidopsis (Li-Cor 6400-17, Li-Cor Inc., Lincoln, NE, USA) connected to a gas analyzer system (LI-6400XT; Li-Cor). Carbon fluxes



( $\mu\text{mol CO}_2 \text{ s}^{-1} \text{ cm}^{-2}$ ) were determined at steady state under growing conditions ( $180 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPFD,  $20^\circ\text{C}$ ) and at  $350 \text{ ppm}$  reference  $\text{CO}_2$ .

Transpirational water loss was determined by successive weighting of the pots over 3 d and nights (every 3 h approximately). Evaporation of water from the soil was prevented by sealing the soil surface with four layers of a plastic film. Whole-plant transpiration rate ( $\text{mg H}_2\text{O h}^{-1}$ ) was estimated as the slope of the linear relationship between weight and time, and then expressed per projected rosette area ( $\text{mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ ). WUE ( $\text{g mg}^{-1} \text{ H}_2\text{O}$ ), the amount of dry matter synthesized per unit of water lost, was calculated as the ratio of absolute growth rate during the period of transpiration measurement to transpiration rate. Absolute growth rate was estimated from zenithal images and converted per unit dry mass using LMA.

**Sucrose and leaf ABA content** Plants were harvested at the bolting stage, in the middle of the day, and immediately frozen in liquid nitrogen to determine the sucrose and ABA content. Sucrose content was determined by enzymatic assay as in Gibon *et al.* (2004). Leaf ABA content ( $\text{ng g}^{-1} \text{ FM}$ ) was determined by radioimmunoassay, as previously described in Barrieu & Simonneau (2000). Leaf samples were ground finely under liquid nitrogen, placed in distilled water ( $5 \text{ ml mg}^{-1} \text{ FM}$ ) and immediately warmed at  $70^\circ\text{C}$  for 5 min before shaking at  $4^\circ\text{C}$  overnight. Extracts were then centrifuged at  $16\,000 \text{ g}$  for 10 min at  $4^\circ\text{C}$ , and the supernatant was conserved at  $-20^\circ\text{C}$  and used for radioimmunoassay.

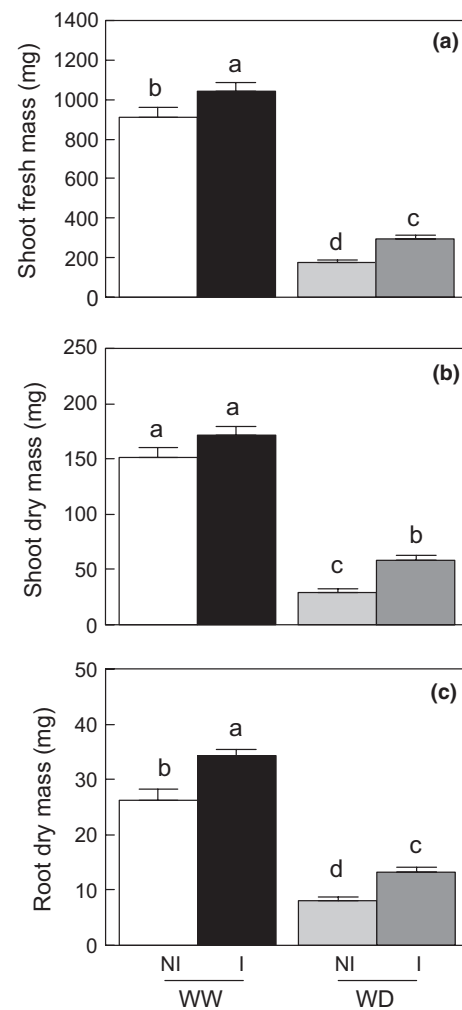
### Statistical analyses

Comparisons of mean trait values between treatments were performed using Kruskal–Wallis nonparametric tests. The effect of inoculation on the phyllochron through time was tested using repeated-measures ANOVA (time treated as random) for each watering condition. All analyses were performed using R 2.15 (R Development Core Team, 2009).

## Results

### STM196 promotes growth of *Arabidopsis Col-0* and increases plant tolerance to water deficit

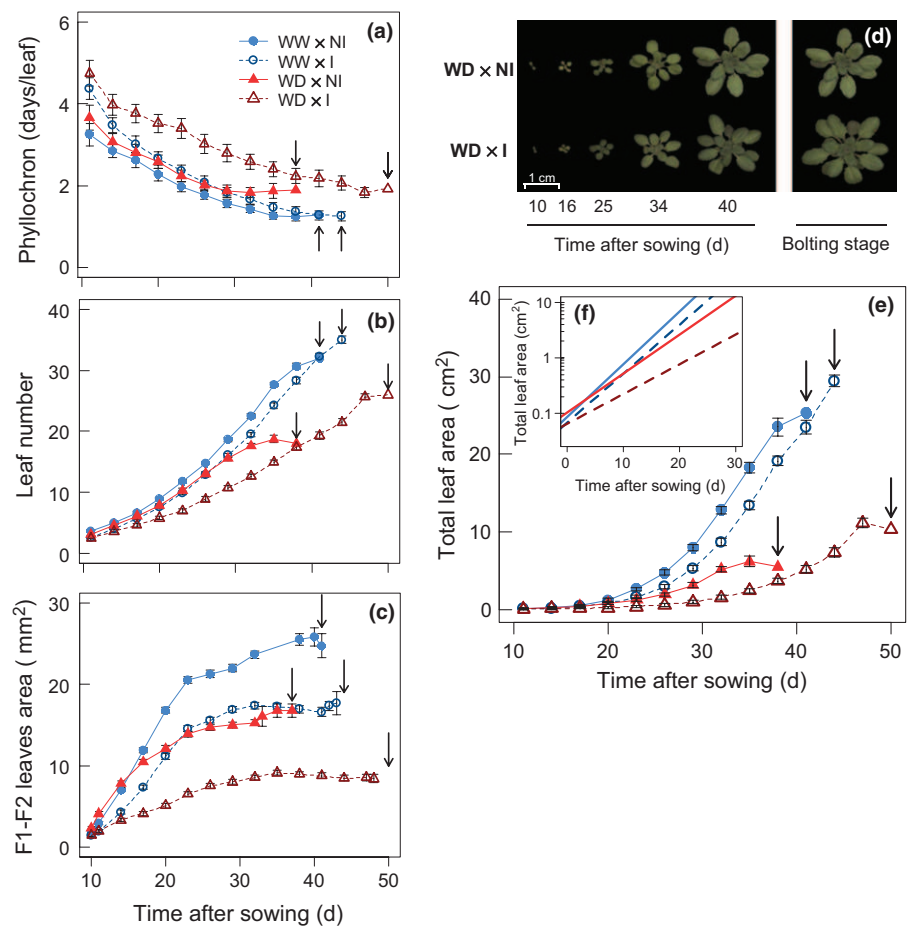
STM196 had a growth-promoting effect on the *Arabidopsis Col-0* under both the WW and WD soil conditions. Under WW, soil inoculation induced a 25% increase of above-ground vegetative FM at emergence of the flowering buds (i.e. bolting stage) (Fig. 1a;  $P < 0.05$ ), but the increase in above-ground vegetative DM was not significant (Fig. 1b). Root DM of inoculated plants increased by 30% (Fig. 1c;  $P < 0.001$ ) under WW. Under WD, inoculation resulted in a larger relative increase in plant size at bolting. First, above- and below-ground DM of inoculated plants were doubled and increased by 67%, respectively (Fig. 1). Secondly, under WD, above-ground FM was reduced by 80% in noninoculated plants but only by 72% in inoculated plants (Fig. 1a).



**Fig. 1** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit on above- and below-ground mass of *Arabidopsis thaliana Col-0*. Data indicate the mean ( $\pm$  SE) shoot fresh mass (a;  $n = 21\text{--}27$ ;  $P < 0.05$ ), shoot dry mass (b;  $n = 8$ ;  $P < 0.05$ ) and root dry mass (c;  $n = 8$ ;  $P < 0.001$ ) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and water deficit (WD) conditions measured at bolting. Different letters indicate significant differences following Kruskal–Wallis test.

### The PGPR strain STM196 induces changes in the developmental dynamics of *Col-0* plants

The effects of soil inoculation by STM196 on the growth and development of *Col-0* appeared very early after germination. A counterintuitive observation is that the growth-promoting effect of STM196 was associated with a delay in the timing of emergence of the first leaves (i.e. inoculated plants had a higher phyllochron; Fig. 2a;  $P < 0.001$  in both watering conditions). The phyllochron of inoculated plants remained higher than the phyllochron of noninoculated plants during the vegetative phase (Fig. 2a;  $P < 0.001$  in both watering conditions) and was not the result of different germination rates between inoculated and noninoculated plants (the mean ( $\pm$  SE) number of d to germination was  $2.83 \pm 0.18$  ( $n = 40$ ) and  $2.71 \pm 0.16$  ( $n = 49$ ) for noninoculated and inoculated plants, respectively;  $P = 0.63$ ). The first two leaves of inoculated plants were also smaller as a result of a



**Fig. 2** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit (WD) on growth and development dynamics of *Arabidopsis thaliana* Col-0. (a–e) Phyllochron (a), leaf number (b), area of the two first leaves (c), representative vegetative phenotypes under WD (d) and total projected leaf area (e) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and WD conditions. Data are means ( $\pm$  SE) of 11–13 plants, except for the area of the two first leaves values ( $n = 9–14$ ). Means of total leaf area and phyllochron are estimated from 3 d intervals around each time point. Arrows indicate bolting time. Insert (f) shows the log-linearized projected area in the exponential phase of vegetative growth.

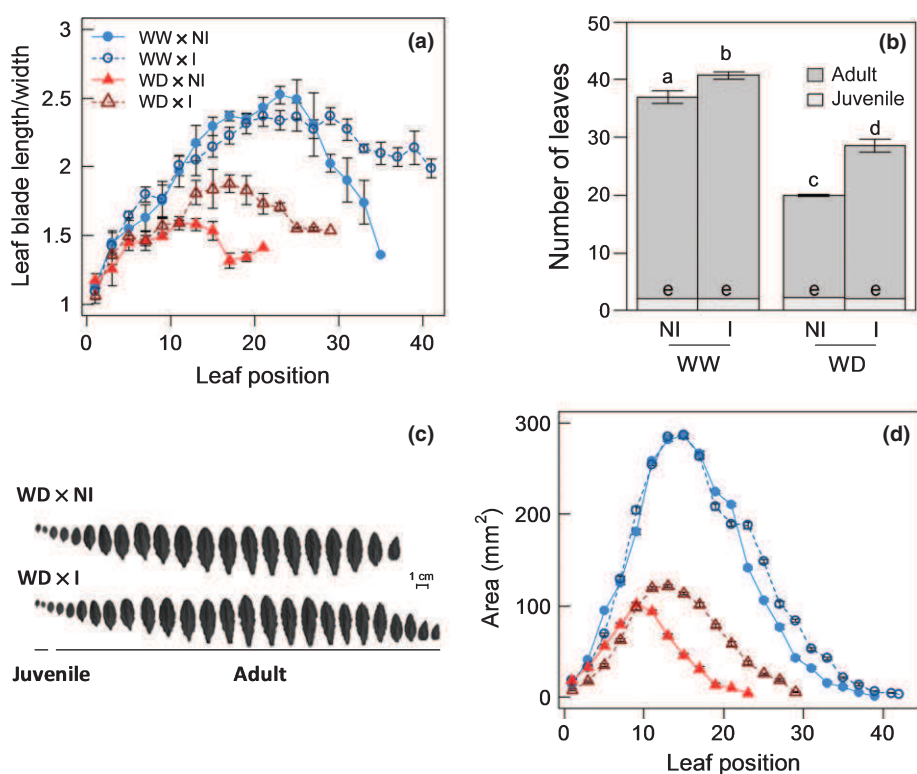
reduced growth rate (Fig. 2c). By contrast, the initial and the maximal relative expansion rates of the rosette (RER and  $R_{max}$ , respectively) were not affected by inoculation (Figs 2f, S3b,d), and therefore inoculated plants remained smaller than noninoculated plants until these latter reached the bolting stage. However, inoculated plants produced more leaves at bolting (Fig. 2b,d), which occurred, on average, 5.5 and 12.5 d later in inoculated plants than in noninoculated plants under WW and WD, respectively (arrows in Fig. 2e).

The delay in the transition from the vegetative to the reproductive phase of inoculated plants was in accordance with the changes in size and shape of the leaves (Fig. 3). The length-to-width ratio of the blades of the first two leaves was close to 1 regardless of the watering condition and the presence of bacteria in the soil (Fig. 3a,c). This ratio then increased rapidly in later adult leaves and decreased at the beginning of bolting stage. The length-to-width ratio was increased for a higher number of leaves in inoculated plants compared with noninoculated plants (Fig. 3a), which indicates that inoculated plants produced a higher number of vegetative adult leaves than noninoculated plants, notably under WD (Fig. 3b,c). Under WW conditions the production of larger leaves occurred beyond the 25th leaf. Under WD, a more pronounced increase in leaf area occurred beyond the 10th leaf (Fig. 3a). As bolting time and the duration of vegetative growth are correlated in *Arabidopsis*, it was not surprising to observe that inoculated plants exhibited a longer duration of rosette area

expansion (Figs 2e, S3c). As a result, despite the developmental slowdown, inoculated Col-0 plants had a higher total leaf area at bolting (Fig. 2d,e), and the larger effect of STM196 under WD compared with WW resulted in a better tolerance to WD.

The developmental slowdown induced by inoculation also led to increased tolerance to water deficit in the early-flowering accession An-1

Next, we investigated the effects of STM196 on the response to WD of the *Arabidopsis* accession An-1, an early-flowering accession. In An-1, flowering stems emerged, on average, 16.4 and 10.8 d earlier than in Col-0 under WW and WD, respectively (solid arrows in Figs 2a, 4a). Inoculation by STM196 did not affect bolting time of An-1, but flowering time was delayed. Flowering time of inoculated plants was delayed by 2 and 5 d compared with noninoculated plants (dashed arrows in Fig. 4a;  $P < 0.05$ ) under WW and WD, respectively. In An-1, shoot FM at bolting was not affected by the presence of bacteria in the soil (Fig. S4a,c). However, STM196 induced a 65% increase of shoot FM and DM at flowering under WD (Fig. S4b,c,  $P < 0.05$ ). Inoculated plants also produced larger flowering stems under WD (Fig. 4d,  $P < 0.05$ ). In this accession, root mass of inoculated plants was significantly increased under WD but not under WW conditions (Fig. S4d). The increase of shoot biomass at flowering was associated with an increase in leaf number and in individual



**Fig. 3** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit (WD) on leaf growth and morphology of *Arabidopsis thaliana* Col-0. (a–d) Length-to-width ratio of the blades (a), number of juvenile and adult leaves (b), representative morphology of rosette leaves arranged from left to right by order of emergence under WD (c) and area of individual leaves (d) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and WD conditions. Data are means ( $\pm$  SE) of eight plants. Different letters indicate significant differences following the Kruskal–Wallis test at  $P < 0.05$ .

leaf area that occurred beyond the 10th leaf (Fig. 4b,c). An-1 plants exhibited the same number of juvenile leaves regardless of the soil treatment, but inoculated plants exhibited more adult and cauline leaves (Fig. 4c).

Hence, under drought, An-1 and Col-0 exhibited similar trends in response to soil inoculation by STM196. The developmental slowdown was characterised by a delayed bolting and flowering time in Col-0, whereas in the early-flowering accession An-1, only flowering time was delayed. In both accessions, the production and expansion of adult leaves were increased in response to soil inoculation by STM196.

### STM196 affects whole-plant physiology and carbon status of *Arabidopsis* Col-0

We measured whole-plant physiology and carbon status only in Col-0 plants, before the reproductive phase. These traits were affected by both the amount of water and the presence of the bacteria in the soil. Transpiration rate was significantly reduced in response to WD in both noninoculated and inoculated plants (Fig. 5a; both  $P < 0.01$ ). Inoculation was also associated with a large decrease in transpiration rate regardless of the watering treatment, especially during the night (Fig. 5a; both  $P < 0.01$ ). This difference in transpiration rate did not result from changes in stomatal density or stomatal index, which were not significantly different among inoculated and noninoculated plants (Fig. S5). LDMC was significantly increased under WD and in inoculated plants regardless of the watering treatment (Fig. 6a,  $P < 0.05$ ). However, the RWC of the rosette was only weakly affected by WD and was not affected by inoculation (Fig. S6;

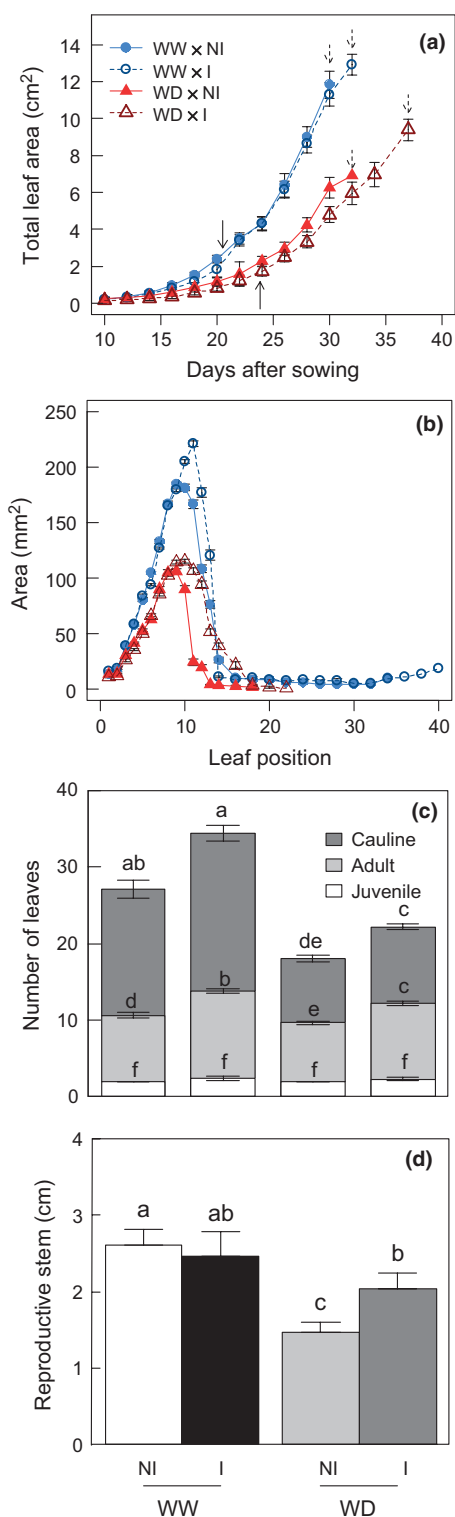
$P < 0.05$ ). Leaf ABA content per unit FM was not significantly affected by the watering treatment but increased in response to bacteria inoculation (Fig. 5b; both  $P < 0.001$ ).

The net photosynthetic rate was not significantly affected by the watering treatment but it was significantly reduced in the presence of bacteria in the soil (Fig. 5c,  $P < 0.05$ ). The leaf carbon status was modified by the watering treatment and the bacteria. Sucrose contents were increased under WD and by the presence of the bacteria in the soil (Fig. 5d;  $P < 0.05$ ).

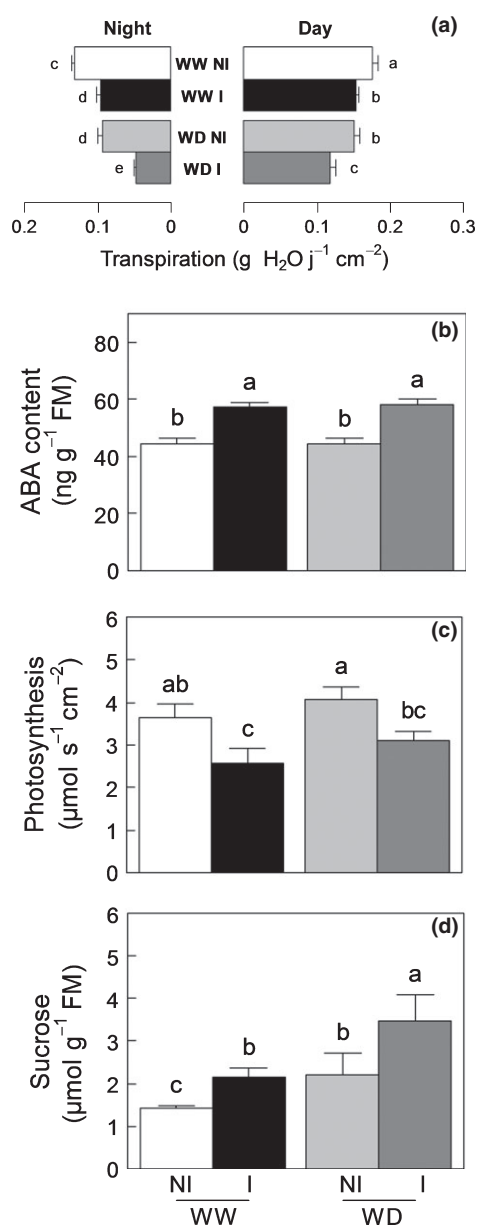
Water-use efficiency of noninoculated plants was not affected by WD (Fig. 6b). WUE was also not impacted by soil inoculation under WW conditions. However, it was significantly increased in the presence of bacteria in the soil under WD (Fig. 6b,  $P < 0.05$ ).

### Discussion

Plant growth-promoting rhizobacteria can enhance plant performance and plant tolerance to environmental stresses by a large variety of mechanisms (for reviews, see Lugtenberg & Kamilova, 2009; Friesen *et al.*, 2011). These mechanisms have to be elucidated to design strategies for PGPR application in agriculture (for review, see Lucy *et al.*, 2004). Here, we show that *Phyllobacterium brassicacearum* strain STM196, a PGPR isolated from the rhizosphere of oilseed rape *B. napus* (Bertrand *et al.*, 2001; Larcher *et al.*, 2003), enhances plant tolerance to drought in two accessions of *A. thaliana* with contrasting flowering phenology. We highlight a new means by which bacteria can enhance plant performance under both well-watered and drought soil conditions. Specifically, our results show for the first time a



**Fig. 4** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit (WD) on growth and development of *Arabidopsis thaliana* An-1. (a–d) Total projected leaf area (a), area of individual leaves (b), number of juvenile, adult and cauline leaves (c) and length of reproductive stem (d) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and WD conditions. Data are means ( $\pm$  SE) of seven to 17 plants. In panel (a), solid arrows and dashed arrows indicate bolting and flowering time, respectively. Different letters indicate significant differences following the Kruskal–Wallis test at  $P < 0.05$ .

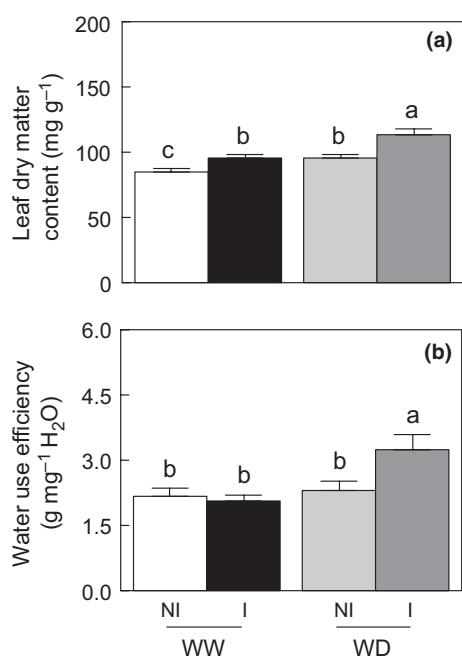


**Fig. 5** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit (WD) on plant physiology and carbon status of *Arabidopsis thaliana* Col-0. (a–d) Night-time and daytime transpiration (a), ABA content in leaves (b), photosynthesis (c) and sucrose content (d) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and WD conditions. Data are means  $\pm$  SE of six to eight plants. Different letters indicate significant differences following the Kruskal–Wallis test.

PGPR-induced delay in the transition from vegetative to reproductive development. Inoculated plants accumulated more biomass before reproduction and exhibited a better WUE.

STM196 induces a delay in reproductive timing that leads to increased biomass accumulation

The switch from vegetative to reproductive development is highly critical for wild and crop species. Indeed, the timing of flowering is a key event that determines the production of plant biomass and therefore yield (Jung & Muller, 2009). Floral transition can



**Fig. 6** Effects of *Phyllobacterium brassicacearum* STM196 and water deficit (WD) on water status of *Arabidopsis thaliana* Col-0. (a, b) Leaf dry matter content (a) and water-use efficiency (b) of inoculated (I) and noninoculated (NI) plants under well-watered (WW) and WD conditions. Data are means  $\pm$  SE of seven to eight plants. Different letters indicate significant differences following the Kruskal–Wallis test at  $P < 0.05$ .

be influenced by abiotic changes in the environment, such as day length, ambient temperature and water availability (Bernier & Perilleux, 2005), and by endogenous stimuli (Huijser & Schmid, 2011). Multiple pieces of evidence support the fact that plant growth rate and the duration of the growth phases depend on flowering time. Alteration of flowering time by genetic modifications or photoperiod resulted in accelerated or decelerated progress towards the vegetative phases of *Arabidopsis* (Steynen *et al.*, 2001) and others species (Salehi *et al.*, 2005). In addition, close relationships among leaf production, individual leaf growth and flowering time have also been reported (Cookson *et al.*, 2007). Here, we showed that *Arabidopsis* plants grown in soil inoculated by STM196 exhibit contrasting growth dynamics and phenology. Developmental changes induced by STM196 appeared very early during plant development. For instance, the phyllochron was increased in inoculated plants as early as the emergence of the two first leaves until the reproductive phase. The most noticeable phenological change was a significant delay in flowering time in inoculated plants. This delay coincided with a prolonged adult vegetative phase, as indicated by the postponed morphological transition between adult and cauline leaves. Moreover, inoculated plants exhibited a prolonged production of adult vegetative and cauline leaves that resulted in a higher number of both types of leaves. Previous studies reported that delaying flowering time as a result of shortening photoperiod coincided with a longer duration of leaf production and growth (Koornneef *et al.*, 1998; Cookson *et al.*, 2007). Here, the prolonged vegetative growth and the delayed flowering of PGPR-inoculated plants led to a greater production of vegetative and reproductive biomass.

Strikingly, the direction of changes was similar in the two watering regimes, but the intensity of changes was more pronounced under drought and led to better plant tolerance to drought. PGPR effects on the timing of flowering are not common and we found no study reporting a PGPR-induced delay in flowering time. Growth promotion by rhizobacteria is often shown at a given date after germination or inoculation (Ryu *et al.*, 2003; Jaleel *et al.*, 2007; Zahir *et al.*, 2008) and we lack a precise study of their effects on growth dynamics and development. Recently, it has been shown that *A. thaliana* plants inoculated with a naturally associated rhizobacterium, *Pseudomonas* sp., exhibited a faster rate of development – plants reached the floral transition earlier – and were bigger (Schwachtje *et al.*, 2011), indicating that various PGPR strains mediate different plant responses.

Rhizobacteria often induce modifications in phytohormone signalling (for a review, see Yang *et al.*, 2009), which may mediate effects on meristem activity and identity (Hayat *et al.*, 2010). Our results showed that ABA was increased in STM196-inoculated plants. By contrast with other PGPR strains, STM196 is not a high auxin producer (Contesto *et al.*, 2010) and, thus, cannot supply plant roots with extra auxin. However, it has been shown that inoculation with STM196 changed auxin distribution within *Arabidopsis* roots towards apices, which probably explains the positive effect of STM196 on lateral root development (Contesto *et al.*, 2010). It is worth mentioning these effects of STM196 on auxin distribution because this hormone also plays a role in the regulation of leaf and floral initiation and of the position of lateral organs (Reinhardt *et al.*, 2000). In addition, other hormonal pathways are modified by STM196, including ethylene, which participates in root hair elongation *in vitro* (Contesto *et al.*, 2008; Galland *et al.*, 2012). Further investigations are required to disentangle the interactions between signalling pathways that might explain the developmental changes following STM196 inoculation (Achard *et al.*, 2006).

These results are novel in the context of plant–microorganism interactions and are promising for agronomy. Reducing or eliminating flowering by altering the endogenous mechanisms involved in the flowering pathway is one of the strategies to increase the yield of biomass crops (Jung & Muller, 2009). For instance, overexpression of the *Arabidopsis* floral repressor gene, FLOWERING LOCUS C (FLC), in tobacco resulted in a significant delay in flowering time and a concomitant increase in the biomass yield (Salehi *et al.*, 2005). In vegetative crops such as cabbage (*Brassica oleracea*), early bolting and flowering limit the potential for yield increases (Jung & Muller, 2009). Therefore, manipulation of flowering time through rhizospheric flora can have important applications in stressed conditions, but underlying regulatory genes remain to be investigated.

The growth slowdown of STM196-inoculated plants, superimposed on that of water deficit, contributes to lifetime water economy and to increased drought resistance

Multiple combinations of traits can participate in plant strategies for dealing with drought, including those that allow drought

escape or drought resistance (Verslues & Juenger, 2011). In addition, several soil microorganisms, including PGPR, can represent an added value to these strategies. For instance, some rhizobacteria help plants to maintain a favourable water status under water deficit (Creus *et al.*, 2004), by enhancing the development of the root system (Marulanda *et al.*, 2009). Here, we quantified numerous morphophysiological traits related to plant growth and development in order to decipher the added value of the PGPR STM196 to the drought response strategies of Arabidopsis. The automated phenotyping platform PHENOPSIS allowed the water limitation in the soil to be precisely controlled and maintained from as early as germination and up to the reproductive phase. Steady-state drought as applied here during the whole-plant cycle is highly relevant for the study of plant acclimation to drought (Verslues & Juenger, 2011). Acclimation processes during steady-state drought may reinforce plant resistance to this stress. Here, the soil water deficit was strong enough to cause an 80% decrease in above-ground FM of noninoculated plants at bolting. This biomass reduction is comparable to previous reports using a similar experimental procedure (daily irrigation to a steady-state soil water content) and similar intensities of drought applied to Col-0 (Hummel *et al.*, 2010; Vile *et al.*, 2012) and other accessions (Tisne *et al.*, 2010).

Reduced plant size and total leaf area are common plant strategies to reduce water consumption and therefore drought injury (Tardieu *et al.*, 2011). Indeed, we recently showed that the inherent size of various Arabidopsis ecotypes was negatively related to drought resistance (Vile *et al.*, 2012), and that mutants that cope better with extreme stress often display a dwarfed stature (see references in Skirycz & Inze, 2010). Our results suggest that the growth slowdown of inoculated plants, superimposed on that of water deficit, has contributed to lifetime water economy and to increased drought resistance. In addition, as found in a previous study performed under similar drought scenarios, the reproductive timing under drought tended to occur earlier in Col-0 and later in An-1 (Vile *et al.*, 2012). This illustrates the variability in drought response strategies in terms of reproductive phenology, and contrasts with the generally held view that drought escape is a common strategy of Arabidopsis (Verslues & Juenger, 2011). However, as in well-watered conditions, inoculated plants of both accessions exhibited delayed reproductive timing under drought. Inoculated plants accumulated twice as much biomass and produced more leaves of a larger area before flowering, had bigger reproductive stems and therefore higher expected reproductive yield. Several lines of evidence point to a higher survival and seed production of later-flowering Arabidopsis accessions (Korves *et al.*, 2007). The timing of flowering often correlates with abiotic and biotic stress avoidance, which is frequently scored as a component of yield, for example in maize (Chardon *et al.*, 2004). In addition, quantitative trait loci for adaptation to drought are often related to flowering time loci (Ducrocq *et al.*, 2008). The advantage of a delay is that there is more time to accumulate more mass that can be invested towards seeds (Metcalf & Mitchell-Olds, 2009). Among the drawbacks of such a strategy, plants have to maintain a favourable use of water during a longer period, especially under drought conditions.

We showed that WUE of inoculated Col-0 plants was significantly improved under water deficit. We did not find any significant change in WUE in response to drought in noninoculated plants, in contrast to previous Arabidopsis studies that reported an increase in WUE (Juenger *et al.*, 2005; Aubert *et al.*, 2010). Interestingly, McKay *et al.* (2003) reported that higher WUE was genetically correlated with delayed flowering in Arabidopsis. Here, higher WUE of inoculated plants was mainly a result of a significantly lower water loss through daytime and night-time transpiration, which may reflect a better drought avoidance strategy. However, as reported by Westgate & Boyer (1985), a decrease in transpiration (by stomatal closure) can be followed on a longer timescale by a reduced plant growth rate, as was observed here in inoculated plants. We also reported a decline in photosynthesis in inoculated plants, but the sucrose content in leaves was increased regardless of the soil condition. This is in accordance with the literature reporting an increase in sucrose content in leaves even if CO<sub>2</sub> diffusion is lowered under water deficit (Quick *et al.*, 1992; Hummel *et al.*, 2010). This could be a result of the uncoupling between photosynthesis and growth under water deficit (Muller *et al.*, 2011). Higher concentrations of ABA in the leaves of inoculated plants can explain the lower transpiration rate resulting from stomatal closure. Some bacteria have the capacity to modulate gas exchanges and ABA metabolism. In *A. thaliana*, Zhang *et al.* (2008) interpreted the augmentation of photosynthetic rate in plants inoculated with *Bacillus subtilis* as being the result of decreased ABA concentrations *in planta*. In the common bean, *Paenibacillus polymyxa* and *Rhizobium tropici* coinoculation has been shown to decrease the ABA content in response to WD (Figueiredo *et al.*, 2008). In addition to acting on the biosynthesis of ABA, some pathogenic bacteria can also modify stomatal opening by acting downstream of ABA biosynthesis. This is the case with *Pseudomonas syringae*, which exude coronatine, a substance that inhibits ABA signalling and prevents stomatal closure (Melotto *et al.*, 2006). By contrast, Cohen *et al.* (2009) found a twofold increase in ABA concentrations in *Azospirillum brasilense*-inoculated Arabidopsis. Bacteria-induced increase of ABA content has been proposed to play a role in alleviation of the drought effect in maize (Cohen *et al.*, 2009). Such an observation would be consistent with our results.

Roots also play a key role in WUE and adaptation to drought. Root biomass was higher in STM196-inoculated plants, and modifications of the root architecture as a result of the presence of the bacteria may have enhanced the water absorption capacity. Indeed, studies performed in gnotobiotic conditions showed that STM196 increased lateral root length (Mantelin *et al.*, 2006; Kechid *et al.*, 2013), and the density and length of root hairs (Galland *et al.*, 2012). Both effects must lead to a greater exchange surface with soil and consequently higher water flux through the whole root system up to the leaves.

## Conclusion

Overall, our results show that the PGPR *P. brassicacearum* STM196 induces a suite of developmental and physiological changes that represent a significant added value to the drought

response strategy of *Arabidopsis*. Developmental and early growth slowed down, but prolonged vegetative growth and reduced transpiration contributed to increasing drought resistance and WUE. Prolonged vegetative growth and delayed flowering induced by PGPR are new in the context of plant–microorganism interactions and may be promising for agronomy. Reducing or eliminating flowering by altering the endogenous mechanisms involved in flowering is one of the strategies for increasing crop yield. Delaying flowering time by rhizobacteria inoculation could represent a valuable strategy for increasing biomass yield.

## Acknowledgements

We thank Christine Granier, Bertrand Muller and François Vasseur for comments on previous versions of the present paper; and Myriam Dauzat, Gaëlle Rolland, Alexis Bediee, Frederic Bouvery, Jessica Kok and Crispulo Balsera for help during the experiments. J.B. was funded by the French Ministry of Higher Education and Research.

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### Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Time courses of meteorological parameters.

**Fig. S2** Time course of mean relative soil water content during plant growth.

**Fig. S3** Duration and rate of rosette expansion of *A. thaliana* Col-0.

**Fig. S4** Effects of *P. brassicacearum* STM196 and water deficit on above- and below-ground mass of *A. thaliana* An-1.

**Fig. S5** Effects of *P. brassicacearum* STM196 and water deficit on anatomical leaf traits of *A. thaliana* Col-0.

**Fig. S6** Effects of *P. brassicacearum* STM196 and water deficit on relative water content of *A. thaliana* Col-0.

**Table S1** Replicate numbers per trait and conditions

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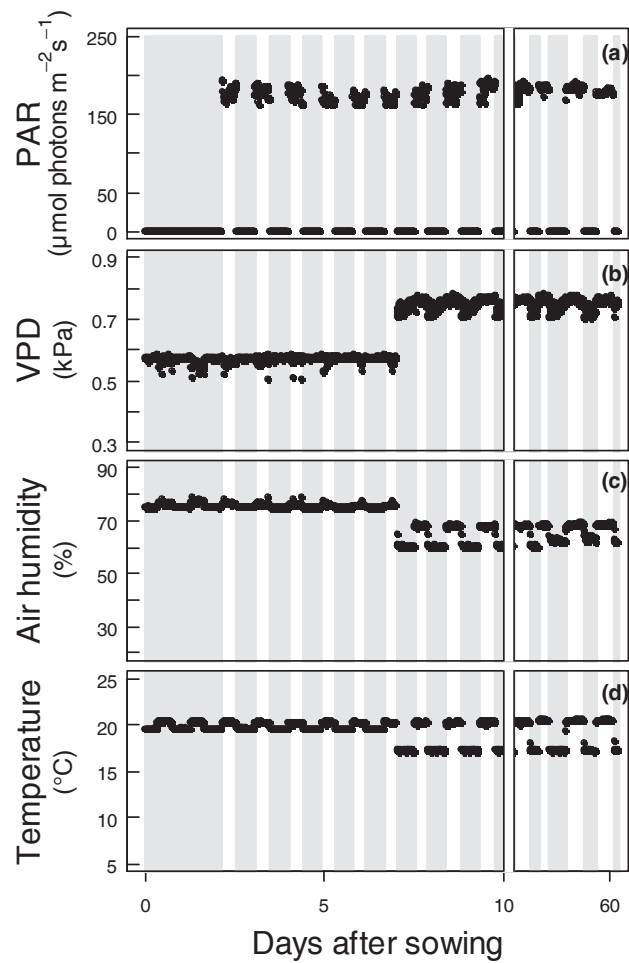
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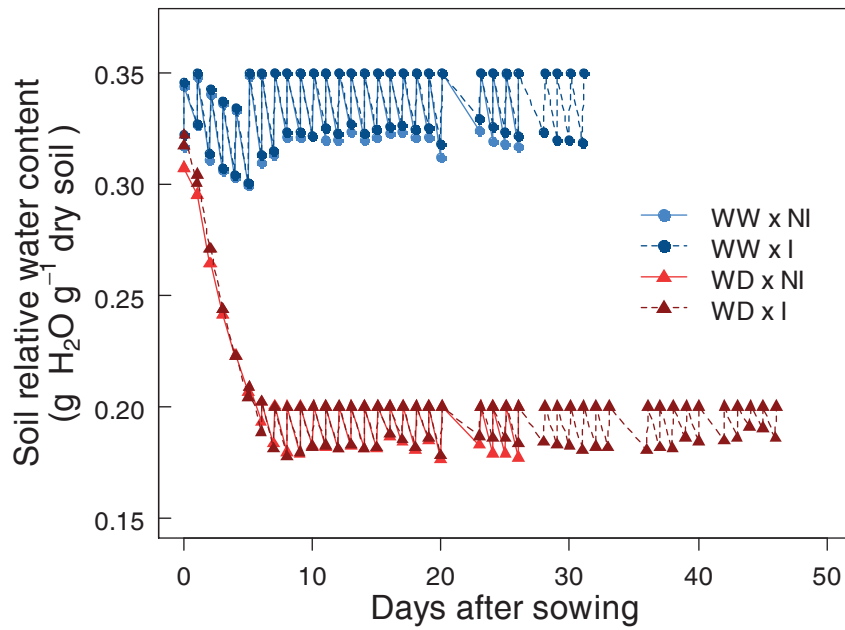
## Supporting information

**Table S1 Replicate numbers per trait and conditions of inoculated (I) and not inoculated (NI) plants under well-watered (WW) and water deficit (WD).**

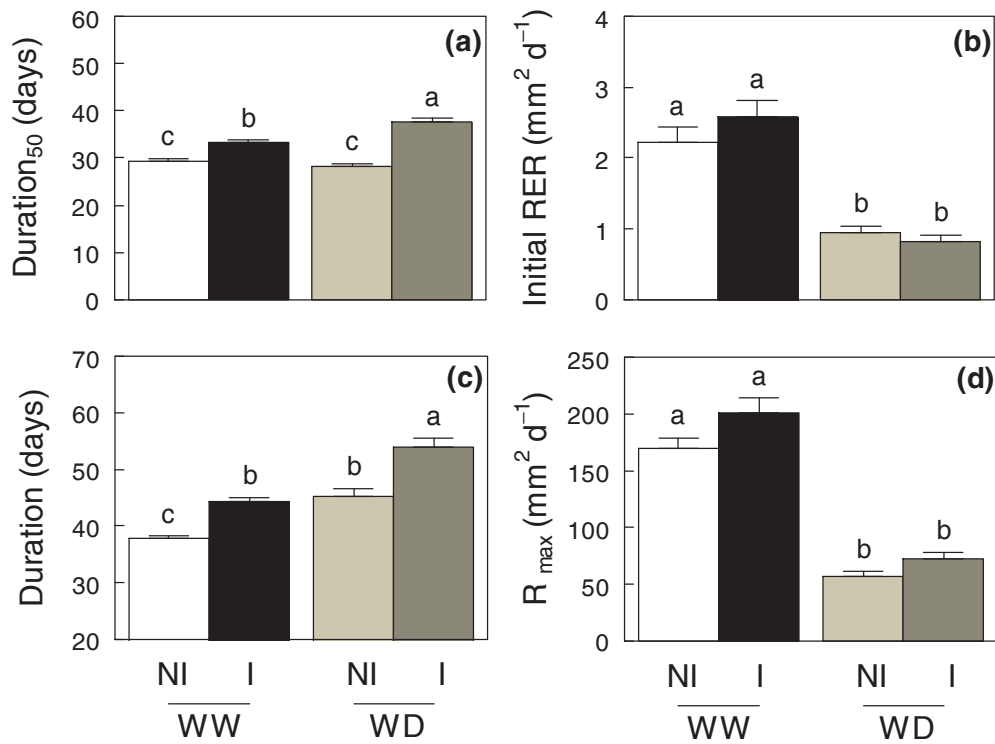
Trait	Col-0 (Bolting)				An-1 (Bolting)				An-1 (Flowering)			
	WW		WD		WW		WD		WW		WD	
	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I
Shoot fresh mass	27	24	24	21	9	12	5	9	8	5	7	7
Shoot dry mass	8	8	8	8	-	-	-	-	8	5	7	7
Root dry mass	8	8	8	8	-	-	-	-	8	5	7	7
Total leaf area	13	13	11	11	-	-	-	-	17	17	11	16
Leaf number	13	13	11	11	-	-	-	-	17	17	11	16
Phyllochron	13	13	11	11	-	-	-	-	-	-	-	-
Individual leaf Area	8	8	8	8	-	-	-	-	8	5	8	7
Leaf blade length/width	8	8	8	8	-	-	-	-	-	-	-	-
F1-F2 leaf area	14	11	9	10	-	-	-	-	8	5	7	7
Reproductive stem	-	-	-	-	-	-	-	-	17	17	11	16
Transpiration	8	8	6	8	-	-	-	-	-	-	-	-
Photosynthesis	8	8	8	8	-	-	-	-	-	-	-	-
ABA	8	8	8	8	-	-	-	-	-	-	-	-
Sucrose	8	8	8	8	-	-	-	-	-	-	-	-
Rosette RWC	8	8	8	8	-	-	-	-	-	-	-	-
LDMC	8	8	7	8	-	-	-	-	-	-	-	-
WUE	8	8	8	8	-	-	-	-	-	-	-	-
Germination Rate	24	23	16	26	-	-	-	-	-	-	-	-
Rmax	13	13	11	11	-	-	-	-	-	-	-	-
Duration	13	13	11	11	-	-	-	-	-	-	-	-
Stomatal density	8	8	8	8	-	-	-	-	-	-	-	-
Stomatal index	8	8	8	8	-	-	-	-	-	-	-	-



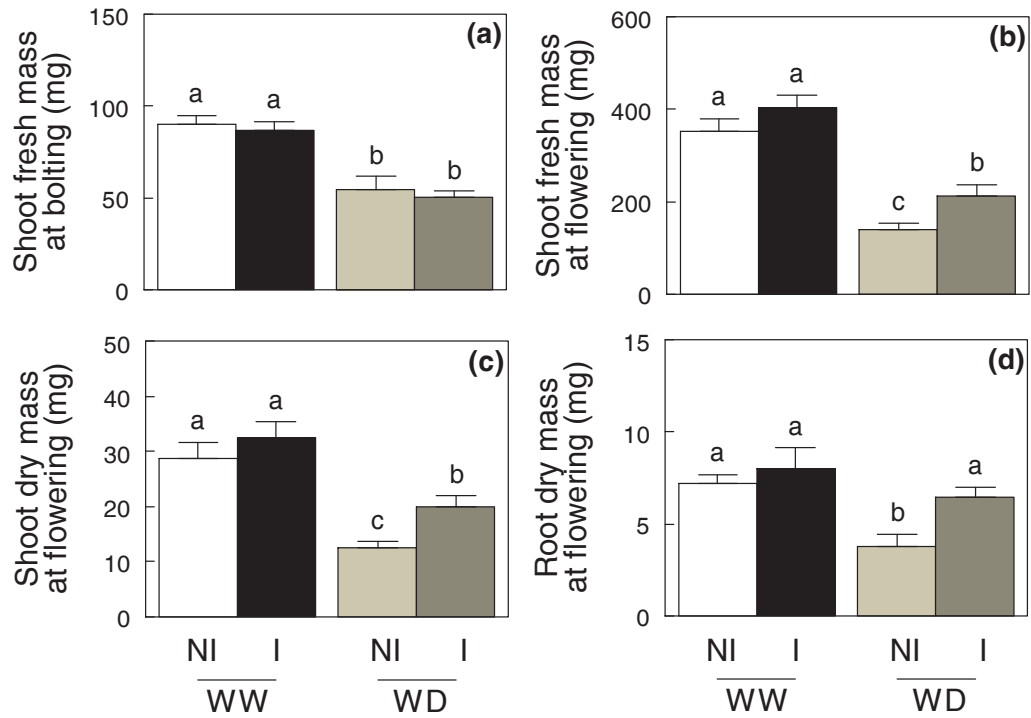
**Fig. S1 Time courses of meteorological parameters.** Photosynthetically active radiation (PAR; a), water vapour pressure deficit (b), air humidity (c) and temperature (d). Each micrometeorological condition was measured with a 10-s time-lapse (Granier *et al.*, 2006).



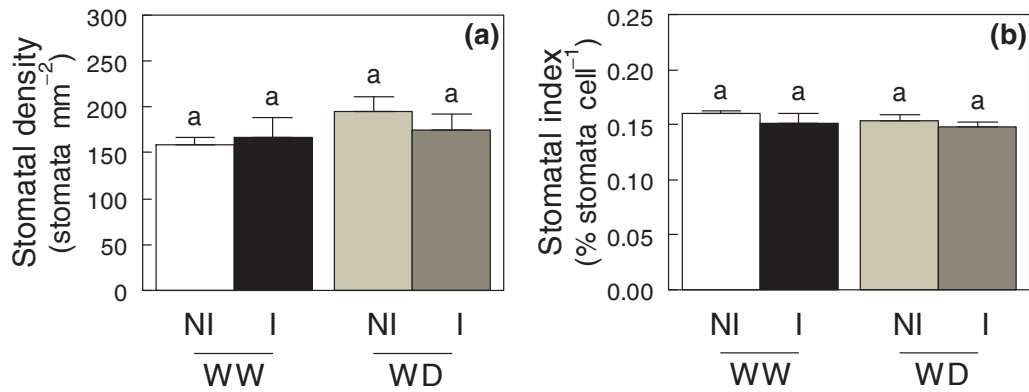
**Fig. S2 Time course of relative soil water content mean during plant growth.** NI: non-inoculated; I: soil inoculated with *P. brassicacearum* STM196; WW: well-watered; WD: water deficit.



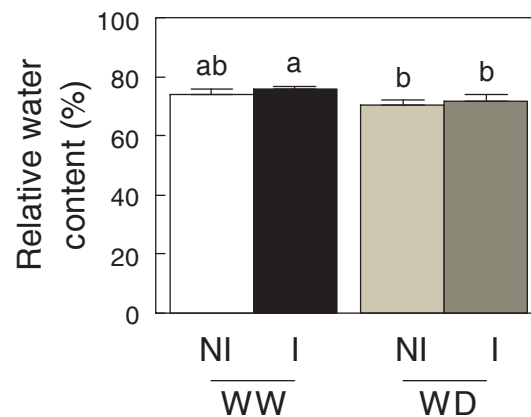
**Fig. S3 Duration and rate of rosette expansion of *A. thaliana* Col-0.** Duration to reach to 50% of final rosette area (a), initial relative expansion rates (initial RER) of the rosette (RER; b), duration of rosette expansion (c) and maximum rate of leaf expansion ( $R_{max}$ ; d) of inoculated (I) and not inoculated (NI) plants under well-watered (WW) and water deficit (WD). Data are mean ( $\pm$  SE) of 11-13 plants. Different letters indicate significant differences following Kruskal–Wallis test at  $P < 0.05$ .



**Fig. S4 Effects of *P. brassicacearum* STM196 and water deficit on above- and below-ground mass of *A. thaliana* An-1.** Shoot fresh mass at bolting stage (a) and at first flower open (b). Shoot dry mass at flowering (c) and root dry mass (d) of inoculated (I) and not inoculated (NI) plants under well-watered (WW) and water deficit (WD). Data are mean ( $\pm$  SE) of 5-12 plants per soil condition. Different letters indicate significant differences following Kruskal–Wallis test at  $P < 0.05$ .



**Fig. S5** Effects of *P. brassicacearum* STM196 and water deficit on anatomical leaf traits of *A. thaliana* Col-0. Stomatal density (a) and stomatal index (b) of inoculated (I) and not inoculated (NI) plants under well-watered (WW) and water deficit (WD). Data are mean ( $\pm$  SE) of 6 plants. Different letters indicate significant differences following Kruskal–Wallis test at  $P < 0.05$ .



**Fig. S6** Effects of *P. brassicacearum* STM196 and water deficit on relative water content of *A. thaliana* Col-0 inoculated (I) and not inoculated (NI) plants under well-watered (WW) and water deficit (WD). Data are mean ( $\pm$  SE) of 8 plants. Different letters indicate significant differences following Kruskal–Wallis test at  $P < 0.05$ .

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## ***Chapitre 4***

# ***Plant-bacteria interaction under severe water stress***

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***Interact to survive:  
Phyllobacterium brassicacearum  
improves Arabidopsis tolerance  
to severe water stress***

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**Short title:** Plant-bacteria interaction under severe water stress

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**Keywords:**

Plant growth promoting rhizobacteria (PGPR); *Phyllobacterium brassicacearum* STM196; *Arabidopsis thaliana*; survival; severe water deficit; chlorophyll fluorescence; water status

## Abstract

Plants can interact with mutualistic bacteria that can alter plant phenotypes through physiological and signaling modifications and lead to new plant abilities. Plant growth-promoting rhizobacteria (PGPR) are known to improve plant growth and tolerance to multiple stresses, including drought, salt, heavy metals or pathogens. We investigated the effect of *Phyllobacterium brassicacearum* STM196 strain, a PGPR isolated from the rhizosphere of oilseed rape on plant responses to severe drought. In a previous study, we showed that STM196 improves the resistance of *Arabidopsis thaliana* to moderate soil water deficit. Here, we show that STM196 also increased the survival rate of *A. thaliana* under different scenarios of severe water deficit. We constructed an index of photosynthetic deficiency (IPD) based on the multimodal distribution of maximal quantum yield of PSII photochemistry estimated by fluorescence imaging ( $F_v/F_m$ ) at the rosette level to investigate the effects of drought and of inoculation on the photosynthetic apparatus. IPD values revealed that STM196-inoculated plants tolerated more damages to the photosynthetic tissues than non-inoculated plants. In addition, inoculated plant exhibited a delayed dehydration and a better tolerance to low water status that lead to delayed and reduced plant mortality. Moreover, inoculation by STM196 allowed a better growth recovery of stressed plants which reached a similar biomass at flowering than non-stressed plants. Our results highlight the importance of plant-bacteria interactions in plant responses to severe drought and provide a new avenue of investigations to improve drought tolerance in crops.

## I. Introduction

Drought is a global concern and episodes of severe drought will most probably be more frequent with dramatic consequences on agriculture (Grayson, 2013). Severe water stress greatly reduces plant biomass production and can lead to plant mortality (McDowell *et al.*, 2008). Over the last decade, it has been widely shown that plants can largely benefit from their interactions with soil microorganisms; especially with plant growth promoting rhizobacteria (PGPR) that colonize the rhizosphere of many plants species (Lugtenberg & Kamilova, 2009). The stimulation of growth by PGPR is often associated with lower plant susceptibility to various biotic and abiotic stresses (Selosse *et al.*, 2004; Yang *et al.*, 2009) and there is a growing interest in the use of these rhizobacteria in agriculture (Lucy *et al.*, 2004; Babalola, 2010).

Survival to drought events is found in plants that are able to maintain key cellular functions under severe water stress and recover similar pre-stress values with minimal damages when conditions become favorable again (Lawlor, 2012). The capacity to tolerate low leaf water status, or dehydration tolerance, is widely variable among species (Thomas *et al.*, 2009). The most spectacular adaptation to survive under severe drought is illustrated by resurrection plants (Moore *et al.*, 2009). They display rapid physiological responses and metabolic adjustments (Kranner *et al.*, 2002), and tolerate nearly complete tissue dehydration. Several plants, with the greatest emphasis being placed on the model plant *Arabidopsis*, are being exploited to understanding of the mechanisms that underlie the plant responses to drought (for reviews see Chaves *et al.*, 2003; Lawlor, 2012).

During mild drought or water stress of limited duration, plants that maintain their water status can complete their life cycle with a reduced performance. However, when stress becomes more drastic or prolonged, plants are no longer able to maintain their leaf water potential and leaf damages occur (Verslues *et al.*, 2006) which can lead to a dramatic reduction of biomass production, and even to plant mortality (McDowell *et al.*, 2008). To prevent tissue damages, and remain alive at low leaf water content, many processes and signaling pathways are involved (Farooq *et al.*, 2009). Osmotic adjustments and accumulation of specialized protective osmolytes allow stabilizing cellular structure, such as proline (Gruszka Vendruscolo *et al.*, 2007), glycine betaine (Sakamoto & Murata, 2002) or trehalose (Elbein *et al.*, 2003). One of the most rapid responses to prevent hydraulic failure is stomatal closure. However under severe water stress, stomatal closure can lead to diminish photosynthetic uptake and induce carbon starvation (McDowell, 2011). Drought-induced

senescence of older leaves can contribute to water saving, while allowing the reallocation of nutrient to the younger leaves (Chaves, 1991). However, leaf senescence alters photosynthetic functioning and chlorophyll (Chl) properties (Lim *et al.*, 2007). Chl-fluorescence measurement, based on the status of the photosystem II (PSII), can give a powerful, rapid and minimally invasive tool that indicates plant health (Murchie & Lawson, 2013). In particular, dark-adapted measurement of maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ) provides a useful indicator of plant status under water stress (Woo *et al.*, 2008; Jansen *et al.*, 2009). In general, decrease in  $F_v/F_m$  ratio is associated to leaf damages related to a decrease in photosynthetic PSII efficiency, that may to some extent be reversible (Woo *et al.*, 2008). After a period of water-stress, it has been shown that plants have the capacity to recover progressively, but sometimes incompletely, their photosynthetic potential (Galmes *et al.*, 2007; Xu *et al.*, 2009) and growth (Lechner *et al.*, 2008). During stress, plant growth rate is reduced, even stopped, but leaf epidermal cells retain their ability to expand when conditions become favorable again (Lechner *et al.*, 2008). Leaf growth cessation is related to modifications of cell wall extensibility where cell stiffening can be involved (Peleman *et al.*, 1989; Ingram & Bartels, 1996).

Rhizobacteria can help plants to cope with negative effects of water deficit by global changes of plant functions. Even though a rich literature exists on plant responses to rhizobacteria under water stress (for reviews see Dimkpa *et al.*, 2009; Yang *et al.*, 2009), studies of PGPR effects on plant survival are surprisingly limited. It has been shown that some rhizobacteria can improve survival of plants under water limiting conditions, notably by inducing the accumulation of trehalose content in the plant or by increasing in leaf water content (Suarez *et al.*, 2008; Rodriguez-Salazar *et al.*, 2009). Under less dramatic conditions, some PGPR can improve tolerance to water deficit through i) modifications in phytohormones content and/or signaling, notably in ethylene, auxin and cytokinin (e.g. Figueiredo *et al.*, 2008; Belimov *et al.*, 2009; Liu *et al.*, 2013) ii) enhancement of antioxydase activities like catalase (Kohler *et al.*, 2008) or superoxide dismutase (Wang *et al.*, 2012), iii) changes in plant functional traits such as photosynthetic capacity but also in chlorophyll content (Wang *et al.*, 2012) and in photosynthetic PSII efficiency (Rincon *et al.*, 2008; Heidari & Golpayegani, 2012) or iv) the formation of a biofilm which enhances soil aggregation and improves water stability in soil (Timmusk *et al.*, 2013).

In the present paper, we investigated the survival, growth and physiological responses of *A. thaliana* inoculated with a free-living PGPR, *Phyllobacterium brassicacearum* strain STM196, under scenarios of severe water stresses with rewatering. STM196 belongs to the

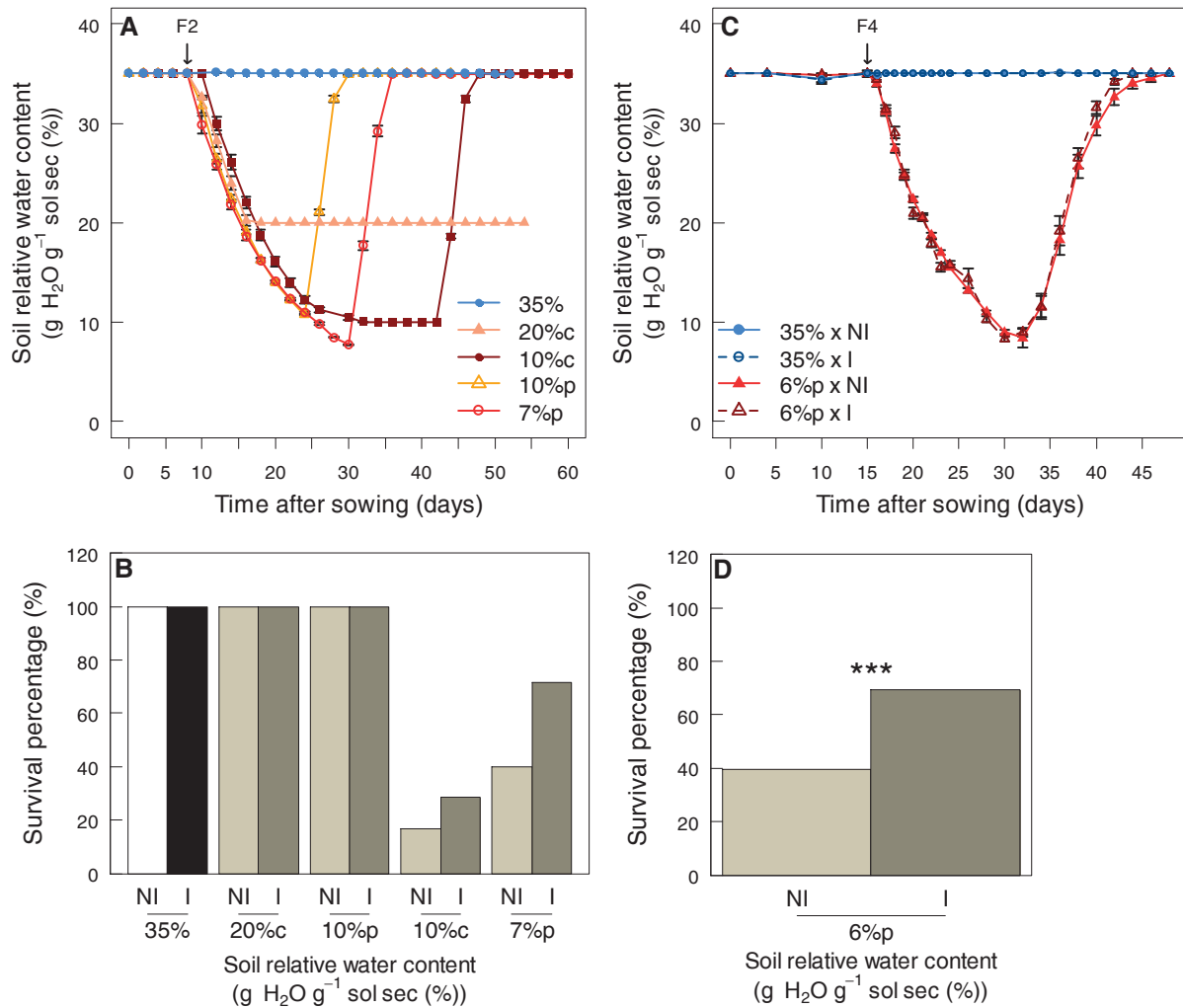
*Phyllobacteriaceae* family in the *Rhizobiales*, order of  $\alpha$ -*Proteobacteria* (Mantelin *et al.*, 2006). This strain was the most efficient PGPR isolated from the rhizoplane of field-grown *Brassica napus* roots (Bertrand *et al.*, 2001; Larcher *et al.*, 2003). We have recently shown that STM196 improves *A. thaliana* resistance to moderate water deficit through reproductive delay and physiological changes (Bresson *et al.*, 2013). Moreover, previous *in vitro* studies showed that STM196 modifies root architecture and hormonal signaling (Mantelin *et al.*, 2006; Contesto *et al.*, 2010; Galland *et al.*, 2012; Kechid *et al.*, 2013). Here, we characterized the responses of *A. thaliana* Col-0 to severe drought using high throughput fluorescence imaging implemented in the plant phenotyping platform PHENOPSIS (Granier *et al.*, 2006). We quantified photosynthetic performance, water status and growth of plants inoculated with STM196 or not.

First, inoculation by STM196 strain greatly improved survival of *A. thaliana* under different scenarios of soil water availability. Then, we developed an index of photosynthetic deficiency (IPD) based on the multimodal distribution of the variable fluorescence ratio ( $F_v/F_m$ ) at the rosette level. Our results suggest that improvement of plant survival by STM196 was related to a better tolerance to higher levels of IPD, indicating that inoculated plants can survive with more leaf damages, and to a delayed dehydration of tissues. In addition, STM196 induced a better recovery of growth following rewatering, leading to a production of biomass similar to non-stressed plants.

## II. Results

### II.1. *Phyllobacterium brassicacearum* STM196 strain increases *A. thaliana* survival under multiple scenarios of severe water deficit

Plants of *Arabidopsis thaliana* Col-0 were grown under five different scenarios of soil water availability in order to determine a level of stress that induced plant mortality and then analyze the effects of STM196 strain on plant survival (see the description of water deficit treatments in SI Table S1). Under well-watered conditions, *i.e.* soil relative water content ( $RWC_{soil}$ ) maintained at 35%  $g\ H_2O\ g^{-1}$  dry soil (corresponding to a soil water potential of  $-0.07\ MPa$ ), all plants survived and reached the reproductive stage (Fig. 1A,B). All plants also survived a continuous moderate water deficit (Fig. 1A,B), *i.e.* irrigation withdrawn from two first leaves emerged and  $RWC_{soil}$  then maintained at 20%  $g\ H_2O\ g^{-1}$  dry soil (corresponding to a soil water potential of  $-0.28\ MPa$ ) until flowering. Decreasing  $RWC_{soil}$  punctually to 10%  $g\ H_2O\ g^{-1}$  dry soil (10%p;  $-3.19\ MPa$ ) did not affect plant survival, but when this  $RWC_{soil}$



**Fig.1. *Phyllobacterium brassicacearum* STM196 increases *A. thaliana* Col-0 survival under severe water deficit.** (A) and (C) represent different water availability scenarios where irrigation was stopped from the two first leaves and four leaves, respectively. Survival percentage was assessed under well water condition (35%, relative soil water content), a moderate and continuous soil water stress (20%c) and four severe and punctual stresses (10%c, 10%p, 7%p and 6%p). 10%c and 10%p were different in their timing to stay at 10% of soil water content before rewatering. (B) and (D) Survival percentage of inoculated (I) and non-inoculated (NI) plants under those different soil drying scenarios.

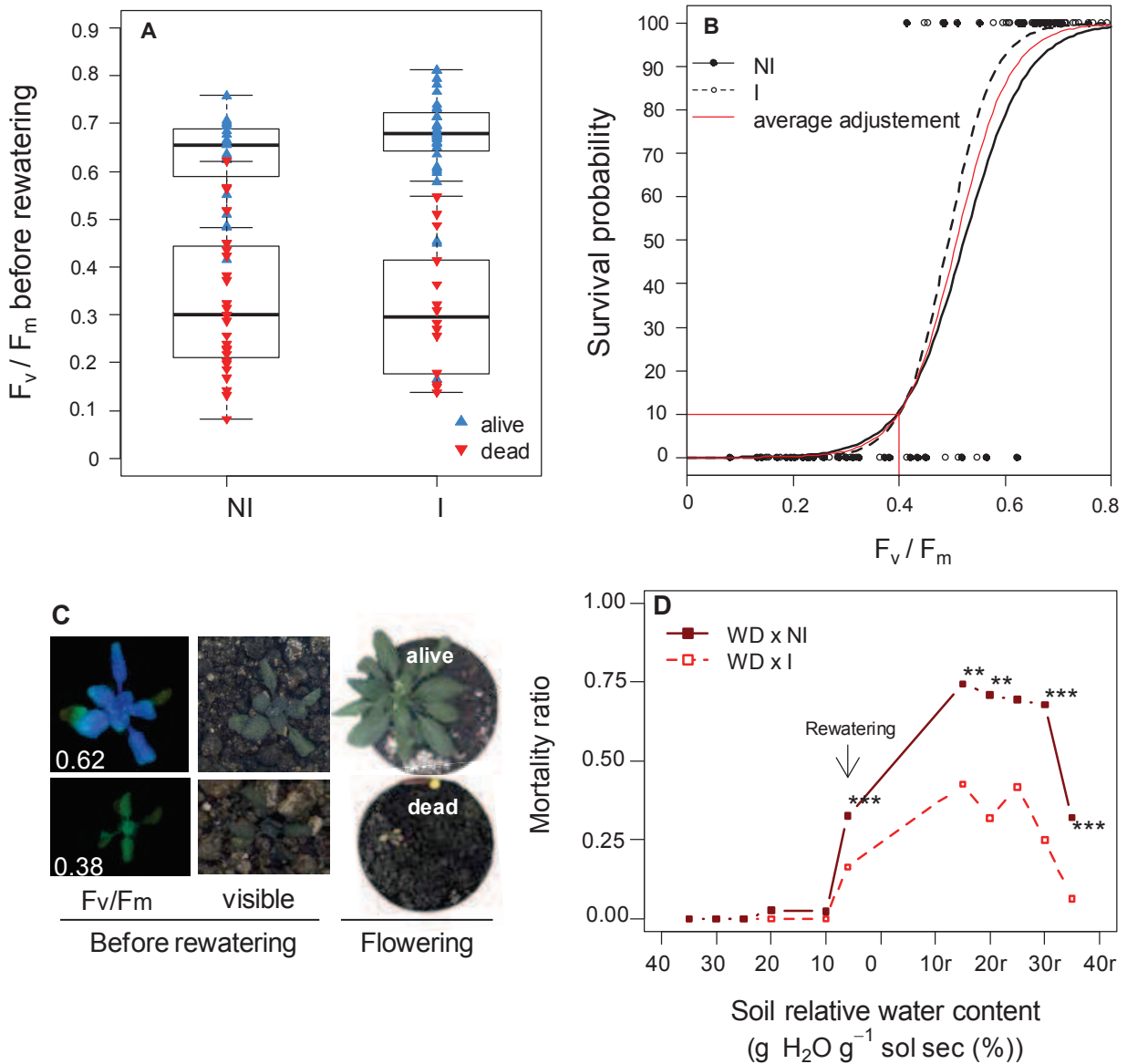
level was prolonged for more than 10 days (10%c) more than 80% of the non-inoculated plants died (Fig. 1A,B). Decreasing  $RWC_{\text{soil}}$  punctually to 7%  $\text{g H}_2\text{O g}^{-1}$  dry soil (7%p;  $-9.52$  MPa) resulted in 40% of non-inoculated plants that survived and reproduced after stepwise rewatering to well watered conditions (Fig. 1A,B).

In order to perform accurate measurements of plant development and physiology during soil drying, the beginning of water stress was delayed to four leaves emerged, and  $RWC_{\text{soil}}$  was punctually decreased to 6% (6%p; Fig 1C). Under this scenario, plant survival rate of non-inoculated plants was 40%, *i.e.* similar to the rate observed under punctual 7%  $\text{g H}_2\text{O g}^{-1}$  dry soil (Fig 1B,D). In all watering scenarios causing plant mortality (10%c, 7%p, 6%p), soil inoculation by STM196 strain resulted in a great increase in plant survival rate (Fig 1B,D). For instance, 70% of inoculated plants survived against only 40% of non-inoculated plants under 6%p ( $P < 0.001$ ). This stress level was reached 1.7 days earlier in inoculated plants than in non-inoculated plants (the mean  $\pm$  SE number of days to reach 6%  $RWC_{\text{soil}}$  was  $16.8 \pm 1.9$  ( $n = 50$ ) and  $18.5 \pm 2.2$  ( $n = 48$ ) for inoculated and non-inoculated plants, respectively;  $P < 0.001$ ). To decipher the effects of STM196 at similar  $RWC_{\text{soil}}$  levels, the traits of stressed plants were analyzed and presented independently of time but in function of soil humidity during soil drying and after rewatering.

## II.2. STM196 strain delays and reduces mortality rate under severe water deficit

Non-destructive measurements of chlorophyll fluorescence were used to follow the photosynthetic performance from early developmental stages to the emergence of flowering stem, under well-watered and severe water deficit (only for 6%p scenario) conditions. Minimal ( $F_0$ ) and maximal ( $F_m$ ) fluorescence of chlorophyll *a* were obtained upon illumination of dark-adapted rosettes. The ratio of variable ( $F_v = F_m - F_0$ ) to maximal ( $F_m$ ) fluorescence, *i.e.* chlorophyll fluorescence yield, was used as a sensitive indicator of photosynthetic performance (efficiency of photosystem II). Under well-watered conditions, whole-rosette mean  $F_v/F_m$  was 0.80 during the entire life cycle and was not affected by soil inoculation with STM196 ( $P = 0.574$ ; see Fig. S1). As expected,  $F_v/F_m$  decreased significantly under severe water deficit. Mean  $F_v/F_m$  just before rewatering (*i.e.*  $RWC_{\text{soil}} = 6\%$   $\text{g H}_2\text{O g}^{-1}$  dry soil) was equal to 0.7 for alive plants whereas it was equal to 0.3 for the plants that failed to develop and flower after rewatering for both non-inoculated and inoculated plants (Fig. 2A,C). A lethal  $F_v/F_m$  threshold was then determined, from a logistic regression,





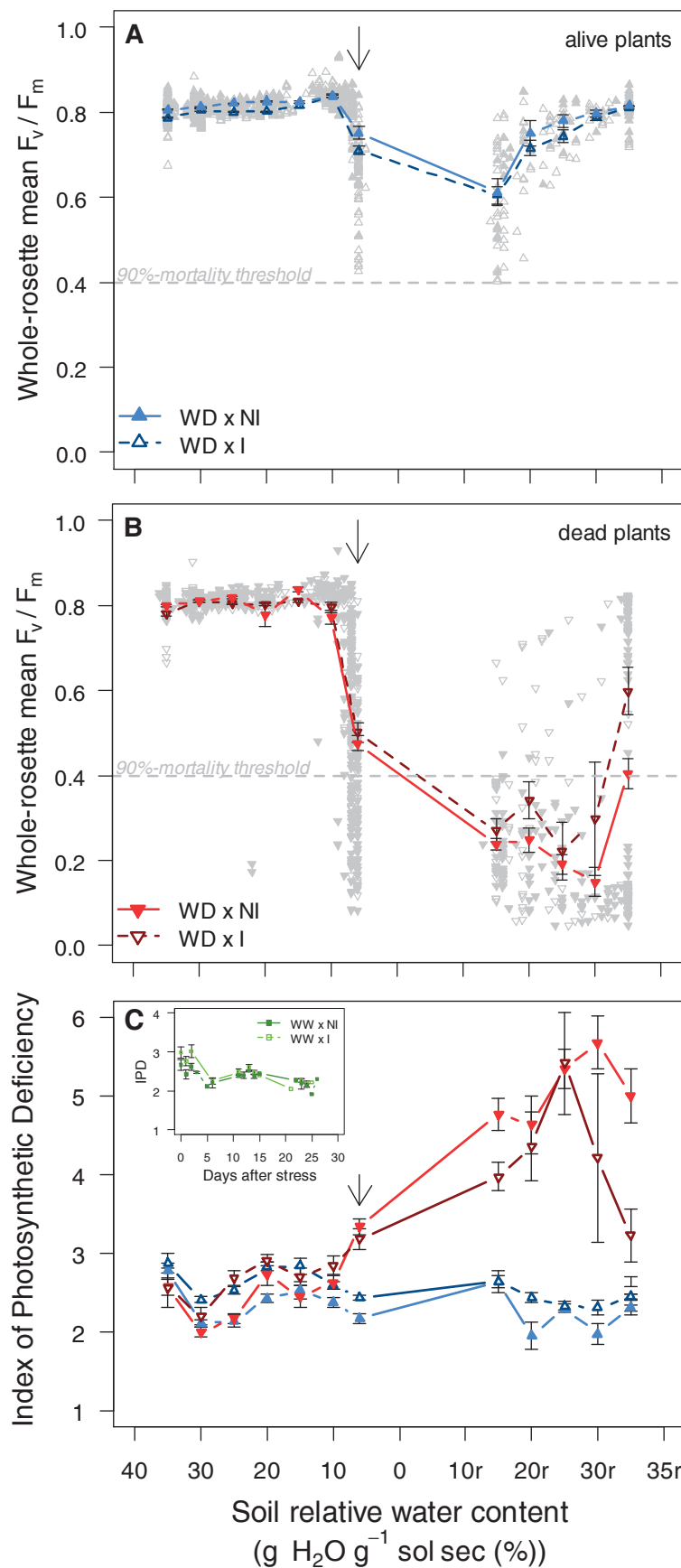
**Fig.2. *Phyllobacterium brassicacearum* STM196 does not affect  $F_v/F_m$ -mortality threshold but delays and reduces mortality ratio during soil drying and rewatering.** (A) Mean  $F_v/F_m$  of alive ( $\blacktriangle$ ; n=19-36) and dead ( $\blacktriangledown$ ; n=16-29) plants non-inoculated (NI) or inoculated by STM196 strain (I) just before rewatering. (B) Estimation of mortality threshold with mean  $F_v/F_m$  of alive and dead plants by logistic regressions. Red dashed line represents 90%-mortality threshold of 0.398 mean  $F_v/F_m$ . (C)  $F_v/F_m$  false-colour images (left) and visible images of vegetative rosette before rewatering (middle) and flowering rosette (right). (D) Estimated mortality rate of stressed plants during soil drying and rewatering of non-inoculated and inoculated plants. Asterisks indicate significant differences following chi<sup>2</sup> test between non-inoculated and inoculated plants (\*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ ).

at 10% survival probability in order to estimate the physiological status of harvested plants during water stress (Fig. 2B). There was no difference between the parameters of the regressions performed on non-inoculated and inoculated plants and the average fit was therefore used (Fig. 2B). The 90%-mortality threshold was inferred at  $F_v/F_m = 0.398$ . In further analyses, plants with  $F_v/F_m$  values above this threshold were considered as able to survive the stress imposed (alive plants) and plants with  $F_v/F_m$  values below this threshold were considered as dead. The distinction between alive and dead plants was crucial to avoid errors of interpretation of the results due to a higher number of inoculated alive plants, and could help to differentiate the behavior of surviving and perishing plants.

Using this threshold to differentiate dead and alive plants during soil drying showed that estimated mortality rate (i.e. proportion of estimated dead plants) tended to increase at 20% g H<sub>2</sub>O g<sup>-1</sup> dry soil in non-inoculated plants and never before 6% g H<sub>2</sub>O g<sup>-1</sup> dry soil in STM196-inoculated plants (Fig. 2D). From RWC<sub>soil</sub> = 6% g H<sub>2</sub>O g<sup>-1</sup> dry soil and after rewatering, the estimated mortality rate of non-inoculated plants was significantly higher than that of inoculated plants (Fig. 2D; note that at the end of the experiment most senescing (decomposing) dead plants were no more detectable, which explain the biased decrease of mortality rate).

### II.3. Delayed dehydration of tissues confers a higher tolerance to photosynthetic damages in STM196-inoculated plants

High-throughput fluorescence imaging allowed the analysis of the distribution of  $F_v/F_m$  values at the rosette level. During water stress establishment (i.e. soil drying), this distribution became heterogeneous and two modes of  $F_v/F_m$  values were gradually distinguishable (bimodal distribution; SI Fig. S2). Upon rewatering,  $F_v/F_m$  values of alive plants rapidly recovered a homogenous distribution (SI Fig. S2). An algorithm for finite mixture models was used to identify the normal density components (i.e. means, standard deviations and mixing proportions) of the mixture distribution of  $F_v/F_m$  values for each individual rosette. At the whole-rosette level, the decrease in mean  $F_v/F_m$  was not progressive in plants exposed to stress but was dramatically affected beyond 10% RWC<sub>soil</sub> both in both alive and dead plants with a higher magnitude for the latter (Fig. 3A,B). At the maximum of stress severity (i.e. 6% RWC<sub>soil</sub>), lowering of whole-rosette mean  $F_v/F_m$  was slightly more pronounced in alive inoculated plants than alive non-inoculated plants ( $P < 0.05$ ), and  $F_v/F_m$  values of inoculated plants was closer to mortality threshold (see grey points and dashed line

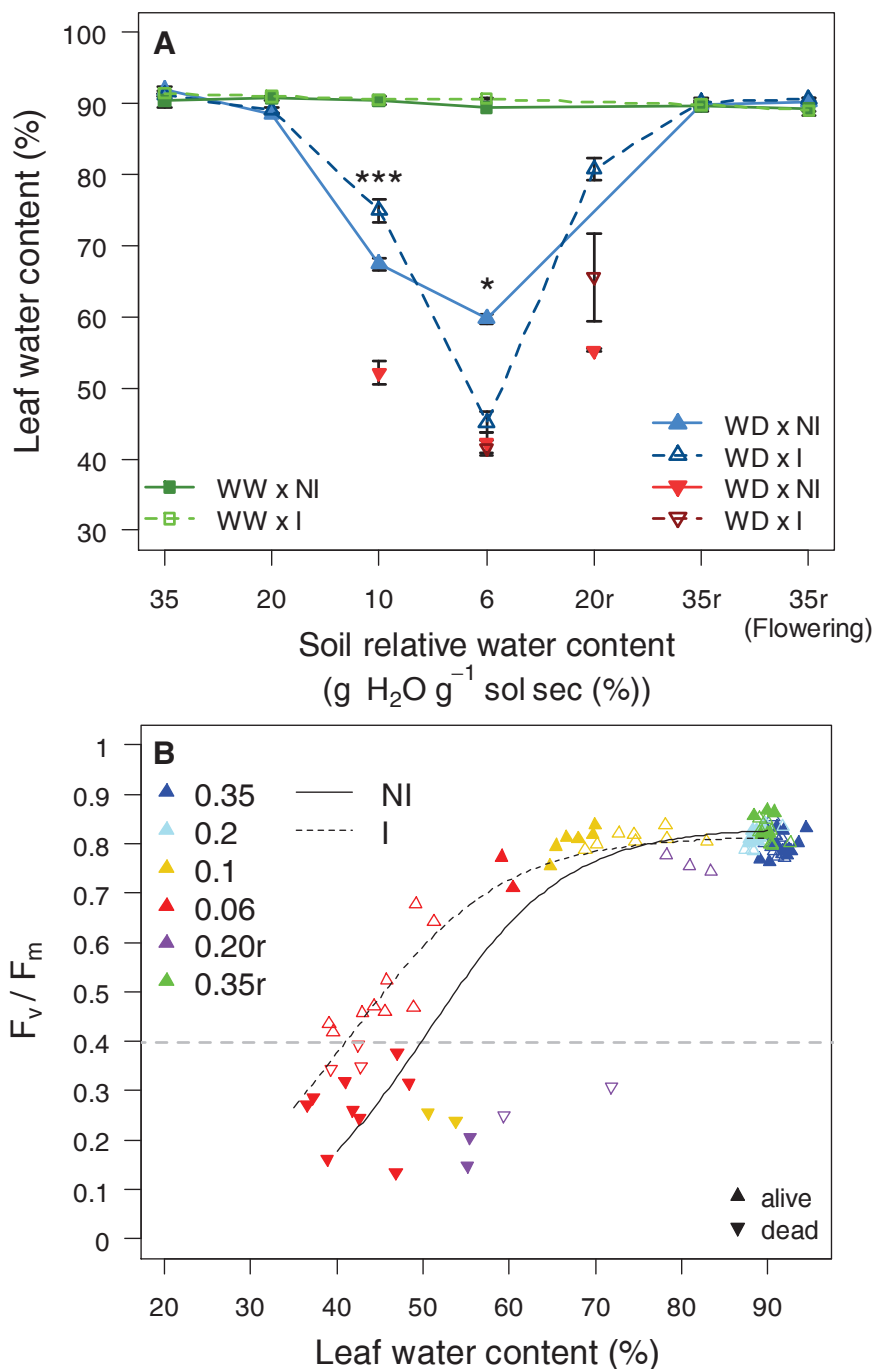


**Fig.3. STM196 improves tolerance to higher levels of Index of Photosynthetically Deficiency (IPD).** (A) Whole-rosette mean  $F_v/F_m$  of alive plants, (B) whole-rosette mean  $F_v/F_m$  of dead plants and (C) IPD of inoculated (NI) and inoculated (I) plants during soil drying (35%, 20%, 10% and 6%) and during rewatering (20r%, 35r% and 35r% at flowering). Insert in (C) represents IPD of plants under well-watered condition (WW) during time after stress (i.e. stage 1.04). Dashed line in (A) and (B) represents 90%-mortality threshold of 0.398 mean  $F_v/F_m$  and arrows indicates rewatering of soil.

in Fig. 3A). Upon rewatering, whole-rosette mean  $F_v/F_m$  continued to decrease and then, both non-inoculated and inoculated alive plants recovered progressively  $F_v/F_m$  values to reach initial mean  $F_v/F_m$  (0.8), similar to non-stressed plants (Fig. 3A and SI). Both inoculated and non-inoculated dead plants reached an equivalent whole-rosette mean  $F_v/F_m$  at 6%  $RWC_{soil}$  (Fig. 3B).

Whole-rosette mean  $F_v/F_m$  was composed of two clusters of pixels grouping in two distributions. The lower values represented the rosette parts with the greatest damages and senescing leaves, whereas less affected parts exhibited healthy regions with a higher fluorescence yield. We constructed an index of photosynthetic deficiency (IPD) that indicated the physiological status of the plants by taking into account the disparity and severity of the decrease in photosynthetic performance during stress. The IPD was calculated using Ashman's D statistic (Ashman *et al.*, 1994) to compare each mode  $i$  (of mean  $\mu_i$ , variance  $\sigma_i$  and weight  $\rho_i$ ) of the mixture distribution of  $F_v/F_m$  values to a hypothetical plant with optimal photosynthetic status (with an unimodal distribution of mean = 1 and variance = 0). Therefore, high values of IPD (caused by a decrease in  $\mu_i$ , and/or an increase in both  $\sigma_i$  and  $\rho_i$ ) indicated that plants were photosynthetically affected and displayed greater leaf damages/senescence. Here, IPD provided a good discrimination between plants exhibiting a 90% probability of survival and plants with a 10% probability of perishing from 6%  $RWC_{soil}$  (Fig. 3C). Moreover, all stressed but surviving plants displayed a similar level of IPD than non-stressed plants, whereas non-surviving (dead) plants exhibited a steep increase in IPD from 6%  $RWC_{soil}$  (insert in Fig. 3C). Surprisingly, during soil drying (i.e. between 35%  $RWC_{soil}$  and 6%  $RWC_{soil}$ ), inoculated plants had higher IPD values than non-inoculated plants for both surviving and non-surviving plants ( $P < 0.05$ ; Fig. 3C). This result suggests that inoculation by STM196 induced a slight decrease in photosynthetic performance but inoculated plants had higher tolerance to photosynthetic damages under stress.

A severe water deficit in the soil unequivocally lead to a reduced water content in plant tissues (Fig. 4A), with deleterious consequences on plant physiology. Plant water status was measured by sequential harvests during soil drying. Leaf water content was progressively affected by soil drying and 6%  $RWC_{soil}$  resulted in a great decrease causing a very low leaf water content compared to plants grown under well-watered conditions (Fig. 4A). At 10%  $RWC_{soil}$ , inoculated alive plants displayed higher leaf water content ( $P < 0.001$ ) than non-inoculated plants, which is the sign that STM196 is able to slow the loss of water in the leaf. At 6%  $RWC_{soil}$  the effect of inoculation was opposite and the leaf water content of alive inoculated plants was lower ( $P < 0.05$ ). This result showed that STM196 allowed plants to



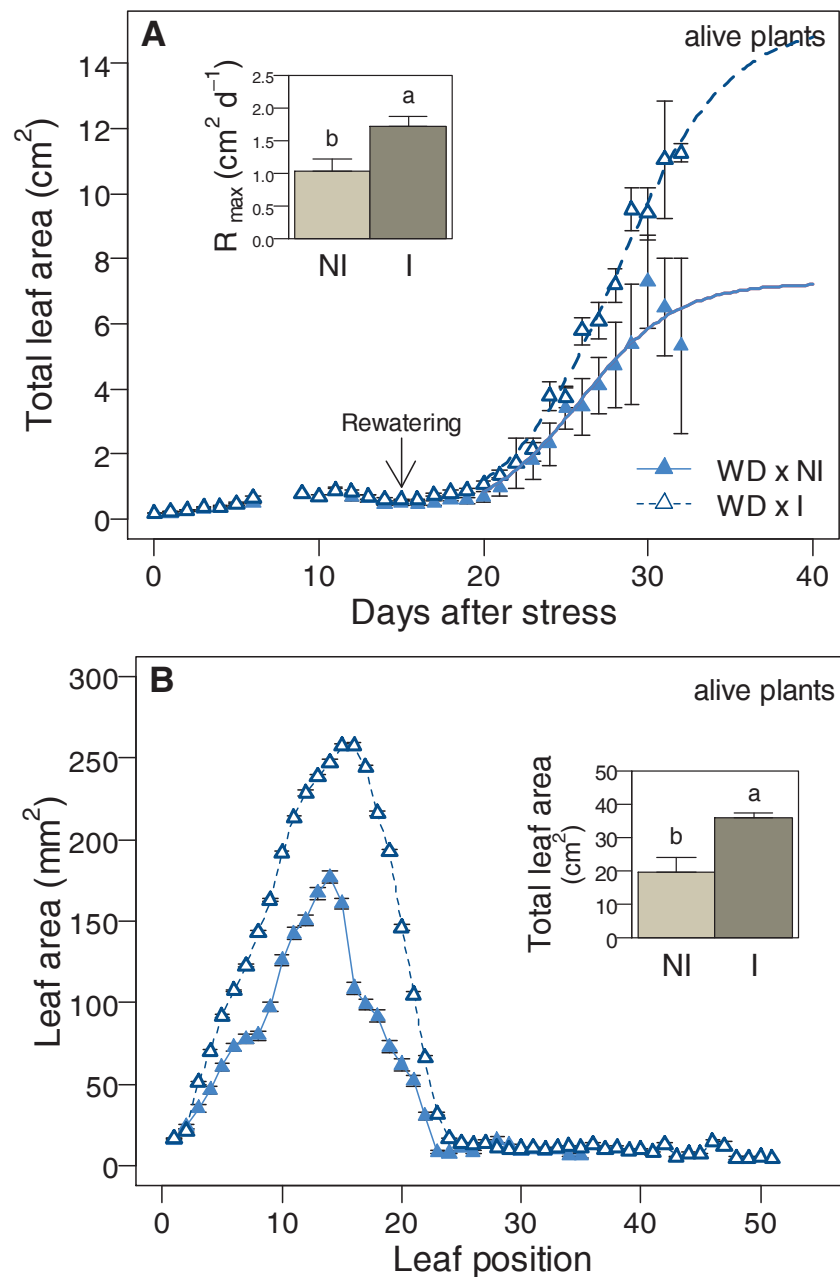
**Fig.4. *Phyllobacterium brassicacearum* STM196 induces a delayed dehydration of tissues.**

(A) Leaf water content and (B) relation between mean  $F_v/F_m$  and leaf water content of inoculated (I) and non-inoculated (NI) plants under well watered (WW) and water deficit (WD) during soil drying (35%, 20%, 10% and 6%) and during rewatering (20r%, 35r% and 35r% at flowering). Flowering was determined by the first flower open. Dashed line in (C) represents 90%-mortality threshold of 0.398 mean  $F_v/F_m$ . Alive plants, with mean  $F_v/F_m$  values, above the threshold, are represented by ▲ points and dead plants, below the threshold, by ▼ points. Asterisks indicate significant differences following Kruskal-Wallis tests between non-inoculated and inoculated plants (\*  $P < 0.05$  and \*\*\*  $P < 0.001$ ).

withstand higher leaf dehydration than non-inoculated plants. Leaf water content and chlorophyll fluorescence were closely related (Fig. 4B). Fitting a logistic regression to the relationship between  $F_v/F_m$  and leaf water content  $WC_{\text{leaf}}$  showed that the decrease of  $F_v/F_m$  in response to water deficit was delayed in inoculated plants compared to non-inoculated plants and appeared for higher values of leaf water content. Together these results showed that STM196 allowed plants to maintain photosynthetic performance at higher leaf dehydration than non-inoculated plants.

#### II.4. STM196 improves growth rate and leaf production recovery of surviving plants, and increases biomass production

Plant growth was modulated by water deficit and growth rate followed the variation of soil water availability. Water deficit establishment resulted in reduced leaf growth, and total leaf area declined until rewatering (Fig. 5A). Upon rewatering, leaf growth of surviving plants resumed and the plants reached the reproductive stage. Soil inoculation by STM196 induced a significant increase in the maximum rate of leaf expansion ( $R_{\text{max}}$ ; Fig. 5A;  $P < 0.01$ ) that led to a larger total leaf area at flowering (insert in Fig. 5B;  $P < 0.01$ ). This was associated with a significant 45% increase of shoot dry biomass (Fig. S3A). The increase in total leaf area of inoculated plants was associated with larger individual leaves than non-inoculated plants (Fig. 5B). At flowering, inoculated plants displayed also a higher number of leaves (Fig. 5B). Flowering time was delayed by 15 days under WD but it was not affected by inoculation (SI Fig S3B). At flowering, all alive plants recovered a complete rehydration of tissues (Fig. S3C). Taken together, all measured physiological and developmental traits had demonstrated a better tolerance of inoculated plants under severe soil water deficit. Moreover, STM196 growth was not affected by water stress and the concentration of bacteria remained constant during the experiment (SI Fig S4).



**Fig.5.** *Phyllobacterium brassicacearum* STM196 increases plant growth rate after rewatering leading to an increase of individual leaf area of alive plants. (A) Total projected leaf area of inoculated (I) and non-inoculated (NI) plants under WD condition during time course. Growth curves were fitted by a logistic regression from rewatering. Insert in (A) represents maximum rate of leaf expansion ( $R_{max}$ ) after rewatering. (B) Area of individual leaves of I and NI plants under WD condition. Insert in (B) shows total leaf area of alive plants at flowering. Flowering was determined at first flower open. Data are means ( $\pm$ SE) of 11-27 plants.

### III. Discussion

Severe water stress induces dehydration of plant tissues and can cause irreversible cellular damages leading to death (McDowell, 2011). Nonetheless, plants are able to some extent to withstand periods in a dried status and restart their metabolic functions after rehydration (e.g. Tyree *et al.*, 2003; Rivero *et al.*, 2007; Lechner *et al.*, 2008). Several genes in *Arabidopsis* have been shown to be implicated in plant survival to water deficit and transgenic modifications could improve plant survival (Skirycz *et al.*, 2011). In addition, some PGPR strains improve tolerance to water deficit, but reports on their effects on plant survival are very scarce, (Suarez *et al.*, 2008; Rodriguez-Salazar *et al.*, 2009) specifically in response to severe water stress in soil.

We recently showed that the PGPR *Phyllobacterium brassicacearum* strain STM196, previously isolated on *Brassica napus*, improved *Arabidopsis* tolerance to moderate water deficit through delayed developmental transitions and modifications of plant physiology. Here, we used the automated phenotyping platform PHENOPSIS that allows the precise control of soil watering (Granier *et al.*, 2006) to analyze the effects of STM196 on *Arabidopsis thaliana* Col-0 response to multiple scenarios of severe water deficit. The scenarios of water deficit used in this study induced a large decrease in plant survival from 60 to 83%, which is comparable to a previous report using a similar procedure (water stress/rewatering from stage 1.04) and similar intensities of soil drying (Skirycz *et al.*, 2011). Plants inoculated by STM196 strain consistently presented a higher survival rate in comparison with non-inoculated plants, through a delayed and reduced mortality rate during water stress establishment. Importantly, STM196 not only increased plant survival but also increased growth recovery in surviving plants and led to a higher biomass production at flowering. Improvement of growth recovery by STM196 could be explained by a better tolerance to stress before rewatering. STM196 also lead to a higher number and larger leaves. This result was in accordance with our previous findings under moderate water deficit (Bresson *et al.*, 2013). Although the precise physiological mechanisms underlying plant mortality are poorly understood, it is well established that severe water stress strongly affects plant growth, water status and causes decline of photosynthetic capacity (McDowell, 2011), specifically through stomatal closure and leaf senescence. Dedicated measurements require a precise knowledge of the dynamics of stress establishment and are often highly time-consuming. For this reason, non-destructive measurements based on chlorophyll fluorescence imaging have been extensively used to decipher the effects of different stresses on plant



physiology (e.g. Gray *et al.*, 2003; Ehlert & Hinch, 2008; Spirdouli & Moustakas, 2012) but have rarely been used at high throughput (but see Jansen *et al.*, 2009). In this paper, we developed a new method of chlorophyll fluorescence analyzes at high throughput in order to decipher the effects of plant-microorganism interactions on plant response to severe water deficit. Amongst the different photosynthetic parameters existing, dark-adapted  $F_v/F_m$ , reflects the maximal efficiency of photosystem II and is therefore one of the most used parameters for measuring leaf physiological status (Murchie & Lawson, 2013). Most often the mean  $F_v/F_m$  of a photosynthetic organ or a whole-plant is used to characterize the response to a stressor. However the image is composed of a panel of pixels whose values range from 0 to 1, and using mean values does not take into account the disparity of  $F_v/F_m$  values across the leaf or the plant. Here, we first showed that the whole-rosette mean  $F_v/F_m$  was significantly related to the probability of survival to severe water deficit. But we also demonstrated that the distribution of  $F_v/F_m$  values may be more informative than the mean ratio to disentangle the dynamics of plant response to stress. Indeed, during establishment of water deficit the distribution of  $F_v/F_m$  became heterogeneous and two distributions can be distinguished. The distribution composed of the higher values represented the healthier parts of the rosette whereas the second distribution with lower values represented the most damaged or senescing parts of the rosette. Exacerbated leaf senescence during prolonged water deficits consistently translates into a large decline in mean  $F_v/F_m$  (Diaz *et al.*, 2005; Wingler *et al.*, 2006). However, senescence is a gradual process that is therefore difficult to quantify in leaves. *Arabidopsis* is a suitable model for the study of leaf senescence since this species produces short-lived leaves and senescence is rapidly induced under stress conditions (Buchanan-Wollaston *et al.*, 2003). Here, we developed an index of photosynthetic deficiency (IPD), based on the gaussian Ashman's D statistic (Ashman *et al.*, 1994), that reflects the proportion of damaged surfaces within a rosette compared to an optimal  $F_v/F_m$  value. IPD gave a more accurate indication of the dynamics of leaf damages induced by the water deficit than the whole-rosette mean  $F_v/F_m$ . IPD was also a good indicator of leaf physiological status because it allowed the discrimination between alive and dead plants, and helped us to unravel the effect of inoculation by STM196 on *A. thaliana* Col-0 survival during severe water deficit establishment.

Plasticity in the timing of senescence may allow acclimation to stressful environmental conditions (Wingler *et al.*, 2006). It has been reported that some PGPR are able to affect photosynthetic efficiency and leaf senescence. Inoculation by the PGPR *Burkholderia phytofirmans* PsJn strain induces a higher number of senescent leaves in *A. thaliana* at

flowering (Poupin *et al.*, 2013). In addition, PGPR can improve photosynthetic activity, especially by an increase of whole-rosette  $F_v/F_m$  values. For instance, inoculation by *Pseudomonas fluorescens* Aur6 strain in *P. halepensis* increases mean  $F_v/F_m$  value that leads to the improvement of tree growth (Rincon *et al.*, 2008). The increase in chlorophyll content could participate to the PGPR-triggered improvement of plant photosynthetic performance (Wang *et al.*, 2012). These modifications lead to growth promotion and a positive correlation between water deficit tolerance and maintenance of efficiency of photosystem II has been observed (Ruiz-Sanchez *et al.*, 2010).

Here, not only inoculation by STM196 significantly increased plant survival to severe water deficit but also it allowed plants to tolerate higher levels of photosynthetic deficiency. Hence, STM196-inoculation induced a better tolerance to damages induced to the photosystem in relation to a delayed dehydration of tissues and an improved tolerance to low water status. Efficiency of photosystem II and leaf water content were tightly related as previously reported by Woo *et al.*, (2008). Traits related to leaf water status are often measured in response to rhizobacteria and drought. In response to PGPR-inoculation, it is widely accepted that rhizobacteria increase leaf water content that leads to increase plant resistance under water deprivation (e.g. Creus *et al.*, 2004; Marulanda *et al.*, 2009; Arzanesh *et al.*, 2011). During water-stress establishment, STM196-inoculated plants displayed a lower decline of  $F_v/F_m$  for a given leaf water content, and non-inoculated plants began to die at lower soil humidity compared to inoculated plants. Moreover, STM196-inoculated plants were more tolerant to low leaf water content. Delayed leaf dehydration induced by STM196-inoculation could explain the delayed mortality. Dehydration delay and dehydration-tolerance are important in survival strategy (Tyree *et al.*, 2003). These could be permitted by osmolytes accumulation, changes in stomatal conductance (ref) and a large and deep root system (Padilla & Pugnaire, 2007). It has been reported that inoculation by *Bacillus spp.* could alleviate negative effects of drought by affecting osmoregulation through increasing proline, sugars and free amino acids (Vardharajula *et al.*, 2011). In the case of STM196 strain, our previous studies under moderate water deficit have shown that inoculation improves *A. thaliana*'s strategy of water saving by a reduced growth rate, a decrease of transpiration rate and an increase of root apparatus (Bresson *et al.*, 2013). Therefore, STM196 may allow a better conservation of leaf water content during stress establishment that could allow a better recovery when soil conditions became suitable for plant growth.

## IV. Conclusion

Overall our findings indicate that inoculation by *Phyllobacterium brassicacearum* STM196 strain reinforced the survival strategy of *A. thaliana* under conditions of severe water stress. STM196 induced a better tolerance to leaf damages through delayed leaf dehydration during water stress establishment that could allow a better growth recovery when soil conditions became favorable again. STM196 remarkably allowed reaching a production of plant biomass similar to non-stressed plants. Improvement of plant tolerance to water stress is a real challenge for crop breeding, specifically under global climate change. The use of plant-bacteria interactions to enhance plant tolerance to abiotic stresses in the field offers valuable and promising prospects in addition or in complement to the classical strategies of genetic selection. Our findings on STM196, and more broadly the use of PGPR, provides interesting avenues in this way, and need to be tested in the field on species with agronomical interest.

## V. Material and methods

### V.1. Bacteria material, bacterial inoculum and soil inoculation

The strain *Phyllobacterium brassicacearum* STM196 was grown for three days in Petri dishes on a sterile (20 min at 120 °C) 1.5% agar (w/v; Sigma-Aldrich) medium (E') containing 2.87 mM K<sub>2</sub>HPO<sub>4</sub>, 0.81 mM MgSO<sub>4</sub>, 1.71 mM NaCl, 7.91 mM KNO<sub>3</sub>, 0.34 mM CaCl<sub>2</sub>, 30 µM FeCl<sub>3</sub>, 1% mannitol (w/v) and 0.3% yeast extract (w/v; Sigma-Aldrich), adjusted to pH 6.8. Next, the bacteria were grown aerobically in liquid E' medium on a rotary shaker (145 rpm) at 25 °C for 24 h to reach the exponential phase of growth. Culture of bacteria cells was pelleted by centrifugation (3200 g, 15 min, 20 °C) and resuspended in water. To obtain 3.10<sup>7</sup> colony forming units (cfu) per gram of soil, the volume was adjusted based upon a correspondence with the absorbance measured at 595 nm (WPA UV 1101, Biotech Photometer, Cambridge, UK). This inoculum was directly put into the soil substrate, which was then manually homogenized.

### V.2. Plant material, growth conditions and irrigation treatments

All experimentations were realized on *A.thaliana* (L.) Heynh accession Col-0. Five seeds were sown at the soil surface in 260 mL culture pots filled with a damped mixture (1:1, v:v) of loamy soil and organic compost (Neuhaus N2) inoculated with STM196 or not. Non-inoculated soil was previously damped with deionized water to avoid difference in initial soil

humidity with inoculated soil. Soil water content was controlled before sowing to estimate the initial amount of dry soil and water in each pot. The pots were kept in the dark during two days in the PHENOPSIS growth chamber (Granier *et al.*, 2006) and were damped with sprayed deionized water three times a day until germination. Then, plants were cultivated under 12 h day length ( $180 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density, PPFD, at plant height). During germination phase, air temperature was set to 20 °C day and night, and air relative humidity was adjusted in order to maintain constant water vapor pressure deficit (VPD) at 0.6 kPa. Then, plants were grown at 20/17 °C day/night and 0.8 kPa of VPD. Soil relative water content ( $\text{RWC}_{\text{soil}}$ ) was maintained at  $0.35 \text{ g H}_2\text{O g}^{-1}$  dry soil until beginning of water stress. After given stage (SI Table S1), seedlings were thinned to one to four plants per pot and soil water deficit was started. Soil water content was maintained at  $0.35 \text{ g H}_2\text{O g}^{-1}$  dry soil in the well-watered treatment (WW) and it was decreased progressively to a desired  $\text{RWC}_{\text{soil}}$  (SI Table S1) by stopping irrigation in the water deficit treatment (WD). For punctual stresses, after reached to a given  $\text{RWC}_{\text{soil}}$ , irrigation was restarted to reach the control soil water content by daily adding a constant volume (10 ml) of a modified one-tenth-strength Hoagland solution (Hoagland & Arnon, 1950). For continuous water stress,  $\text{RWC}_{\text{soil}}$  was maintained during plant life cycle. Control soil water content was maintained until harvests at first flower open (stage 6.00 Boyes *et al.*, 2001).

Survival percentage was scored in three consecutive experiments that were carried out following the same experimental procedure (see SI Table S1). In experiment 1 and 2, different scenarios of water availability were performed and water stress was started at stage 1.02. In order to maximize the statistical power experiment 3 was performed with high repeat number by conditions and to make physiological measurement water stress was started at stage 1.04 and  $\text{RWC}_{\text{soil}}$  was decreased to reach  $0.06 \text{ g H}_2\text{O g}^{-1}$  dry soil.

Soil water potential was determined by using a potentiometer (WP4-T dewpoint meter, Decagon Devices, Pullman, WA 99163, USA) during the drying of soil (from  $0.35$  to  $0.06 \text{ g H}_2\text{O g}^{-1}$  dry soil, Fig. S5)

### V.3. Dynamic measurement of plant traits during soil drying scenario

Different measurements were performed during establishment of water stress and after rewatering at different points of  $\text{RWC}_{\text{soil}}$ :  $0.35$ ,  $0.20$ ,  $0.10$  and  $0.06 \text{ g H}_2\text{O g}^{-1}$  dry soil during soil drying and  $0.20\text{r}$ ,  $0.35\text{r}$  after rewatering.

### V.3.1. Measurement of photosynthetic efficiency

The maximum quantum yield of PSII was determined by  $F_v/F_m$  measurement on dark adapted plants, after 8-12 hours of dark, by using an IMAGING-PAM chlorophyll fluorometer (Walz; Effeltrich, Germany) with W- IMAG-K6 camera type, and ImagingWin software application connected with PHENOPSIS.  $F_v/F_m$  is given by the equation:  $F_v/F_m = (F_m - F_0)/F_m$  (Maxwell & Johnson, 2000). The measurement was initiated by exposing leaf to measuring light pulses at 1 Hz frequency, Intensity 2, for determination of  $F_0$ . A Saturating pulse (Si 9, width 800ms) was applied to determine  $F_m$  (maximum fluorescence in the dark adapted state).

### V.3.2. Determination of plant water status

Rosettes were cut and immediately weighted after the removal of inflorescence stems to determine aboveground vegetative fresh mass (FM). The rosettes were oven-dried at 65 °C for 48 h, and rosette dry mass (DM) was determined. Water content ( $WC_{\text{leaf}}$ ) was determined by FM/DM ratio.

### V.3.3. Rosette expansion during time course

Projected area of the rosette ( $RA_{\text{proj}}$ ) were determined every days from semi-automated analysis (ImageJ 1.43C, Rasband, Bethesda, Maryland, USA; Fabre *et al.*, 2011) of zenithal images of the plants (Sony SSC-DC393P camera). A sigmoid curve was fitted for each plant following  $RA_{\text{proj}} = a / [1 + \exp[-(d-a/2)/b]]$  where  $a$  is the maximum area, and  $d$  is the number of days after sowing. The maximum rate of leaf expansion ( $R_{\text{max}}$ ,  $\text{mm}^2 \text{d}^{-1}$ ) was calculated from the first derivative of this logistic model at  $d_0$  as  $R_{\text{max}} = a/(4b)$ .

## V.4. Whole-plant and leaf morphology at flowering

Surviving individuals were harvested at first flower open. Rosettes were cut and immediately weighted after the removal of inflorescence stems to determine aboveground vegetative FM. The rosettes were wrapped in moist paper and placed into Petri dishes at 4 °C in darkness overnight to achieve complete rehydration. Water-saturated fresh mass (SM) was then determined. Total leaf number was determined, and the leaf blades were separated from their petiole and scanned for measurements of leaf area (ImageJ 1.43C; Rasband, Bethesda, MD, USA). Leaf blades, petioles and reproductive structures were then separately oven-dried at 65 °C for 48 h, and their dry mass was determined. Rosette DM was calculated as the sum of blades and petioles dry masses. From these measurements, relative water content ( $RWC =$

$(FM - DM) \times 100 \times (SM - DM)^{-1}$ ) was calculated at the rosette level. All phenotypic data are stored in the PHENOPSIS database (Fabre *et al.*, 2011).

### V.5. Quantification of bacteria in soil

To analyze bacterial growth under water stress, a natural mutant of STM196 strain was selected in a selection medium E' contains  $100 \mu\text{g ml}^{-1}$  of rifampin and then, was transformed by use of pCH60 vector. The vector pCH60 encodes for tetracycline resistance and contains *gfp* gene that is constitutively expressed (Cheng & Walker, 1998). The bacterial growth was followed in soil by use of the *gfp*-tagged and rifampin-tetracycline-resistant STM196 strain in the same condition of plant growth culture. Measurement of bacterial concentration was performed at 0.35, 0.20, 0.10 and  $0.06 \text{ g H}_2\text{O g}^{-1}$  dry soil during soil drying out and at the end of experiment (at  $0.35 \text{ g H}_2\text{O g}^{-1}$  dry soil after rewatering). Quantification of bacteria was performed in soil without of plant. The concentration of colony-forming units (cfu/mg) was determined by use an estimate called the most probable number (MPN) (Halvorson & Ziegler, 1933). 100 mg of inoculated soil taken from the plant rhizosphere were put in 1 ml of physiological water ( $8.5 \text{ g l}^{-1}$  de NaCl) on a rotary shaker (145 rpm) at  $25 \text{ }^\circ\text{C}$  for 2 h 30. The solubilized soil samples were serially diluted until  $10^{-7}$ , and  $100 \mu\text{l}$  were spread in Petri dishes on a sterile (20 min at  $120 \text{ }^\circ\text{C}$ ) 1.5% agar (w/v; Sigma-Aldrich) medium (E') with addition of  $50 \mu\text{l}$  ml of rifampin and tetracycline. Bacteria were counted after 6 days at  $25 \text{ }^\circ\text{C}$ .

### V.6. Statistical analyses and determination of the Index of Photosynthetic Deficiency (IPD)

All analyses were performed using R 2.15 (R Development Core Team, 2009). Comparisons of mean trait values between treatments were performed with Kruskal–Wallis non-parametric tests. Plant survival and mortality ratio were analyzed by  $\chi^2$  tests. A lethal  $F_v/F_m$  threshold was determined by analyze whole-rosette  $F_v/F_m$  just before rewatering in alive and dead plants. Plants that failed to develop and flower after rewatering were considered as dead. Relation between survival probability and whole rosette  $F_v/F_m$  values was modeled by a logistic regression by each soil conditions. Effect of inoculation was tested by  $\chi^2$  tests on deviance ratio. The 90%-mortality threshold was inferred from regression at 0.398 of  $F_v/F_m$ . Plants with  $F_v/F_m$  values above this threshold were considered alive and plants with  $F_v/F_m$  values below this threshold were considered as dead. The REBMIX algorithm for finite mixture models (Nagode, 2004) as used to identify the normal density components (i.e.

means, standard deviations and mixing proportions or weight) of the mixture distribution of  $F_v/F_m$  values for each individual rosette. Index of Photosynthetic Deficiency (IPD) was calculated based on bimodal distribution of pixels  $F_v/F_m$  values. We calculated  $D_{\max}$  and  $D_{\min}$  as the Ashman'D of (Ashman *et al.*, 1994), respectively, the maximum mode (max) and minimum mode (min) relatively to the maximum possible  $F_v/F_m$  value (reached without energy loss and technical errors) of mean 1 and variance 0. Thus,  $D_{\max} = |1 - \text{Mean}_{\max}| / (0.5 \times \text{SD}_{\max})^{1/2}$  and  $D_{\min} = |1 - \text{Mean}_{\min}| / (0.5 \times \text{SD}_{\min})^{1/2}$ . Then IPD is calculated as follow  $\text{IPD} = \text{Weight}_{\max} \times D_{\max} + \text{Weight}_{\min} \times D_{\min}$ .

## Acknowledgments

We thank Gaëlle Rolland, Alexis Bediee, Crispulo Balsera, Garance Koch for help during the experiments. We thank also Antoine LeQuéré and Isabelle Babic for provide us *fp*-tagged and rifampin-tetracycline-resistant STM196 strain. J.B. was funded by French Ministry of Higher Education and Research.

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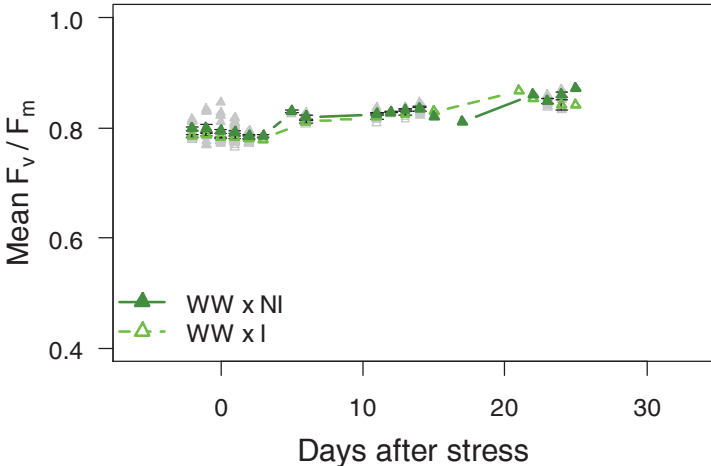
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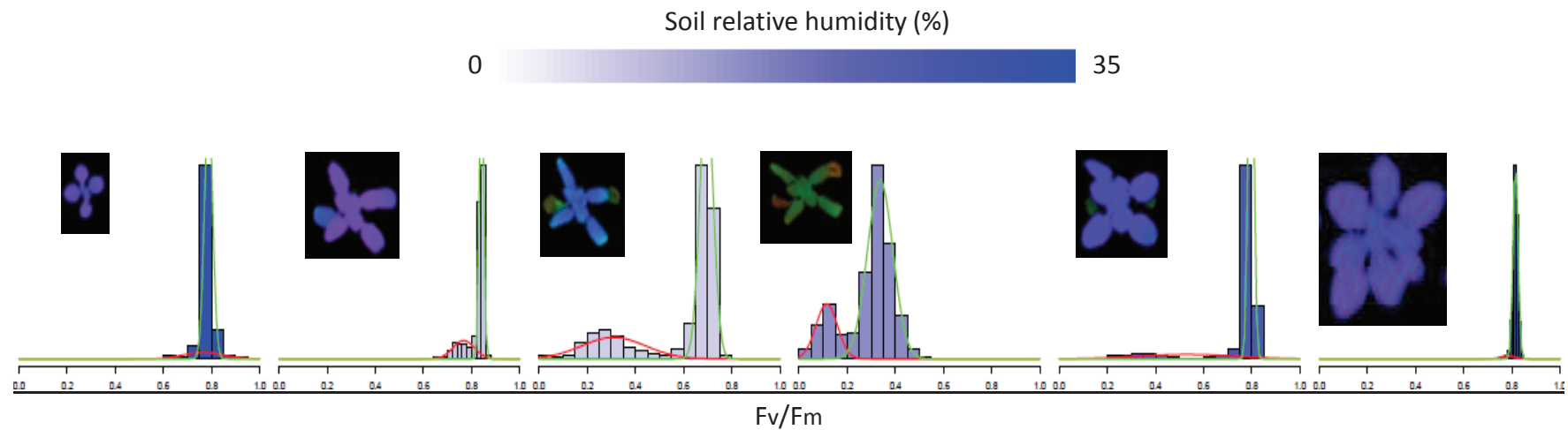
Supporting information



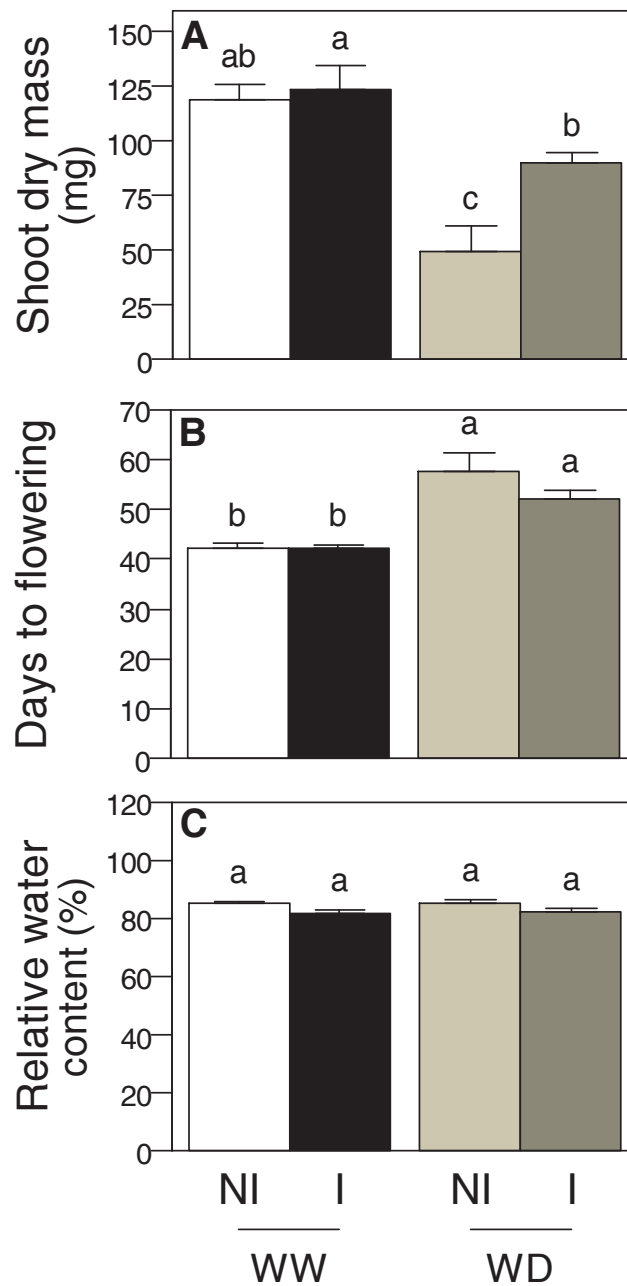
**Fig.S1.** Mean  $F_v/F_m$  is not affected by inoculation under well watered condition (WW). Mean  $F_v/F_m$  of non-inoculated plants (NI) and inoculated plants (I) during time courses.

**Table S1. Description of water availability scenario imposed and design of experiments**

Treatment	Stress scenario	Start of water deficit					Experiment			
		Soil water content (g g <sup>-1</sup> )	Soil water potential (Mpa)	Stage	Days to reach stress after stage	Days to Rewatering	Number of plants per condition	1	2	3
35%	Well-watered condition	0.35	-0.07				13-16	x	x	x
20%	Stop watering and continue soil water content	0.2	-0.28	F2	6		9-20			x
10%	Stop watering, continue soil water content and rewatering	0.1	-3.19	F2	15	32	7-12	x		
10%p	Stop watering and rewatering	0.1	-3.19	F2	14	14	7-12	x		
7%p	Stop watering and rewatering	0.07	-9.52	F2	20	20	7-10	x	x	
6%p	Stop watering and rewatering	0.06	-	F4	30	30	48-52			x

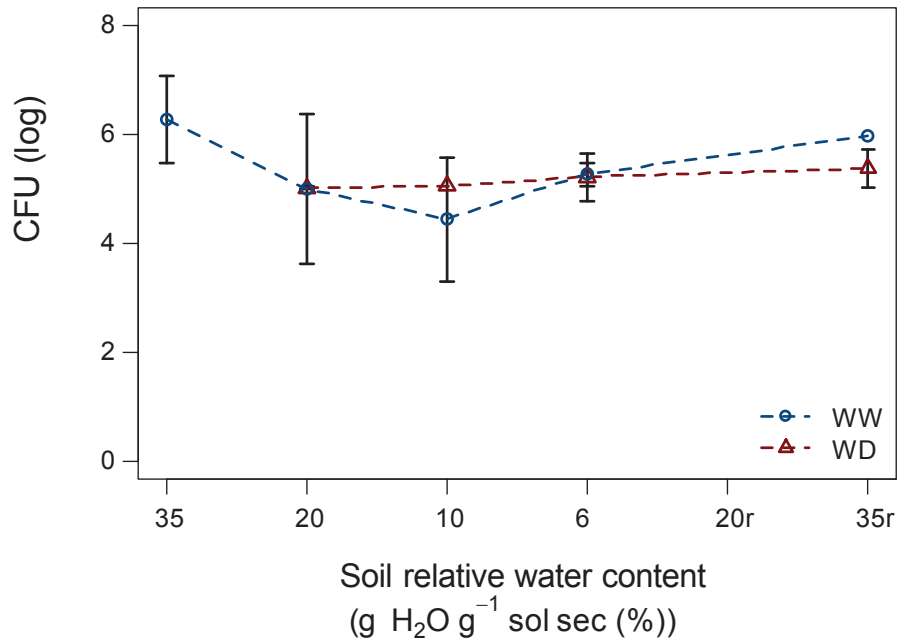


**Fig.S2. Example of the change in the distribution of  $F_v/F_m$  values during stress.** Six distributions of  $F_v/F_m$  values at the rosette level during water stress and rewatering scenario (6%p). From left to right: 35%, 10%, 6%, 10r%, 20r% and 35r% of relative water content in soil (RWCsoil). Pictures of rosettes show  $F_v/F_m$  values (false color). Histograms are colored depending on RWCsoil (corresponding scale above). Green and red lines represent the distributions of maximum and minimum modes from mixture-model, respectively.

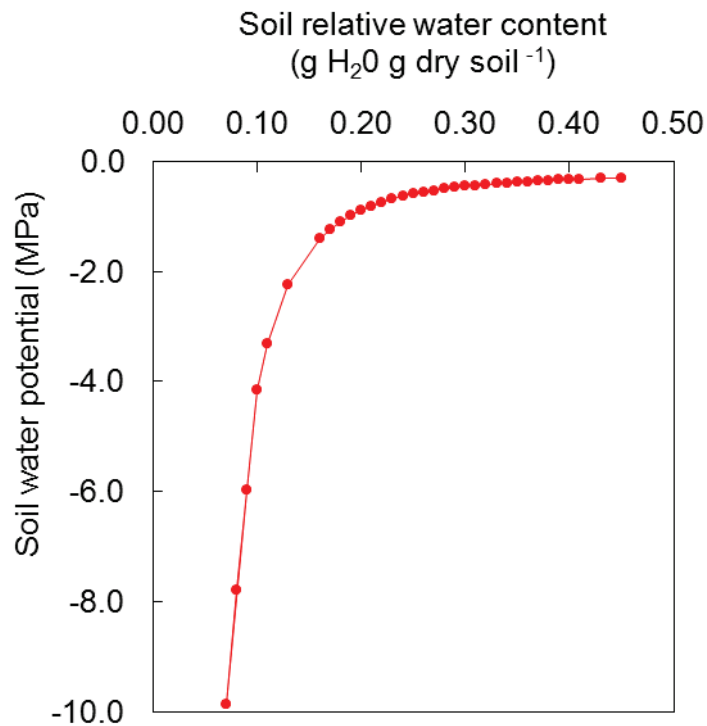


**Fig.S3. Effect of *Phyllobacterium* STM196 strain and water deficit on growth, physiology and development of *A. thaliana* at flowering.** (A) dry fresh mass of leaves, (B) leaf relative water content and (C) days to flowering of inoculated (I) and non-inoculated (NI) plants under well watered (WW) and water deficit (WD). Data are means ( $\pm$ SE) of 11-27 plants. Different letters indicate significant differences following Kruskal-Wallis test  $P < 0.05$ .





**Fig.S4. *Phyllobacterium* STM196 growth does not affected by soil water deficit.** Growth of STM196 strain are represented by cfu/mg of soil under well watering condition (WW) and water stress (WD).



**Fig.S5. Soil water potential during drying of soil**

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## ***Chapitre 5***

# ***Trehalose implication in plant-bacteria interaction***

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# ***Implication of trehalose metabolism in the interaction between the PGPR *P. brassicacearum* STM196 and *A. thaliana****

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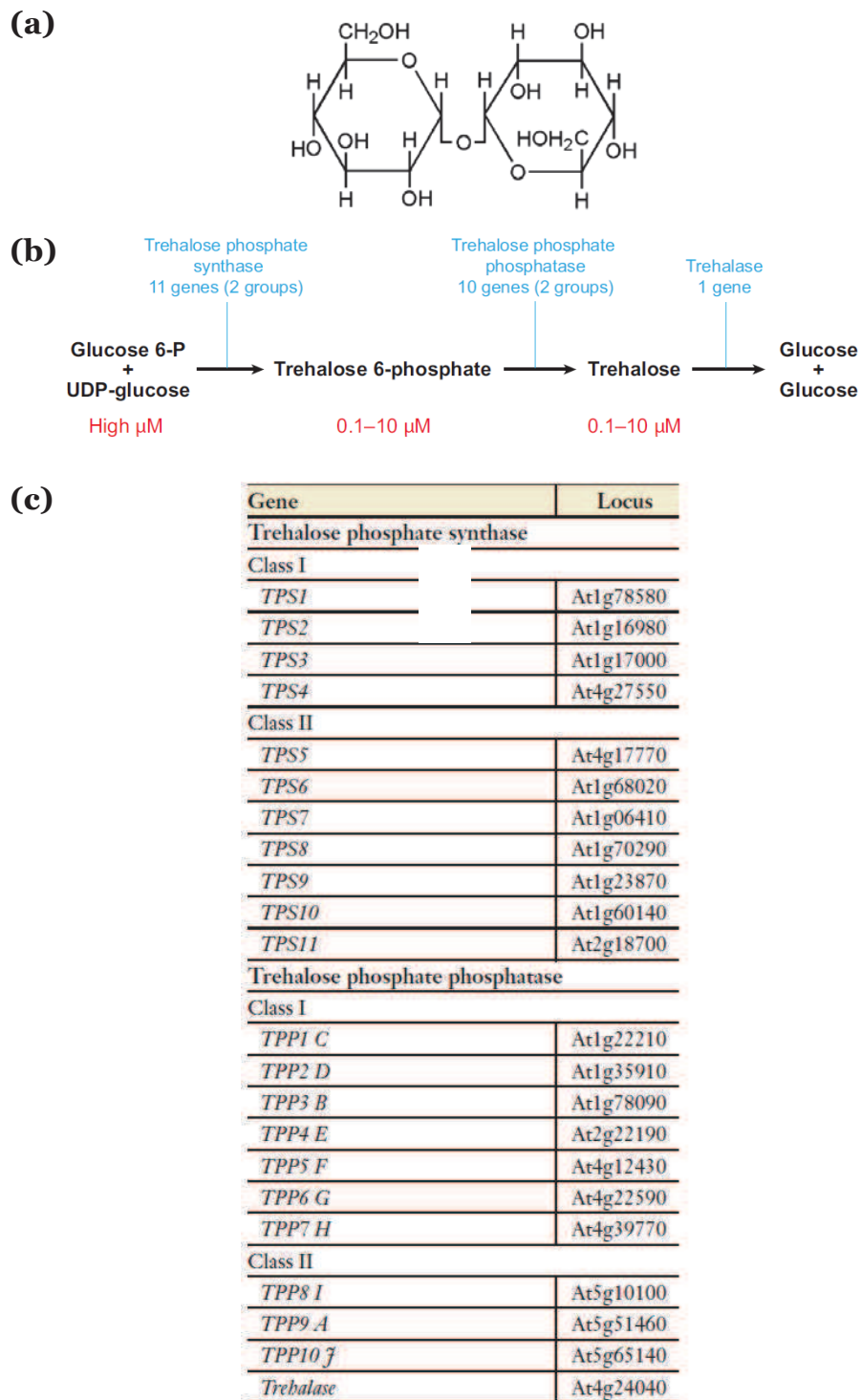
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## ***Abstract***

The use of PGPR is one of the most recent technologies developed to protect crops against various forms of stress. In this study, we hypothesized that trehalose and its precursor T6P are functional molecules in *Arabidopsis thaliana* responses to inoculation by the PGPR *Phyllobacterium brassicacearum* STM196 strain under stress conditions. The implication of trehalose and T6P on plants development and physiology are well documented in the literature. Moreover, trehalose metabolism is undoubtedly involved in plant responses to water deficit. However, the studies of the effects of trehalase, the only known enzyme in trehalose degradation, on plant responses are very scarce. Here, we showed that trehalase-modified mutants displayed strong modifications in flowering time, growth and carbon metabolism, which contrasted with findings in TPP-TPS (genes of trehalose and T6P biosynthesis). Moreover, modified-trehalase expression had opposite effect depending of stress severity and could be negative for plant resistance under moderate stress and positive under more severe stress. Unmodified-trehalase expression was also required for STM196 action on plant responses to moderate water stress. Although it is clear that trehalose metabolism is implicated in STM196-*A. thaliana* interaction, the exact mechanism of its action on plants remained confused.



**Fig.1. Trehalose metabolism in plants (according to Paul *et al.*, 2008)**

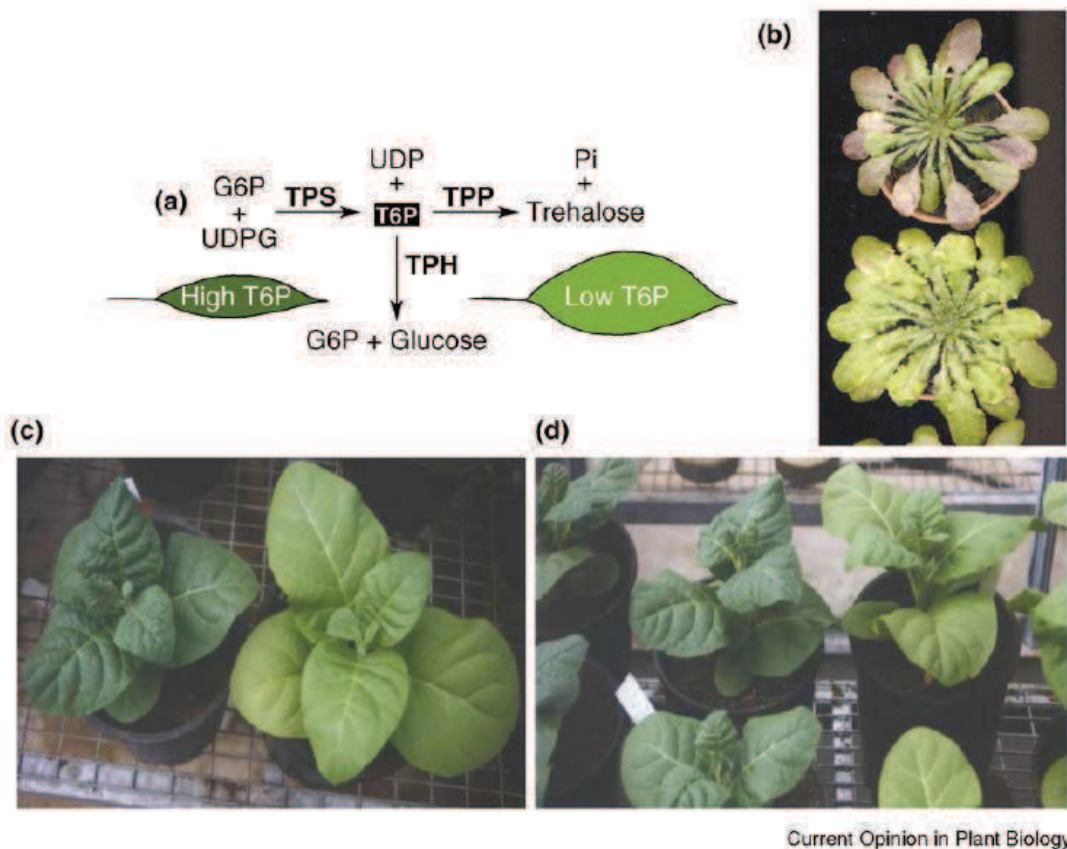
**(a)** Structure of trehalose presented as cyclic Haworth projections. **(b)** Trehalose synthesis and breakdown and **(c)** trehalose pathway genes in *A. thaliana*.

## I. Introduction

Beyond their metabolic roles, recent studies indicate that plant sugars also act as signaling molecules. Sugars could control gene expression and developmental processes in plants (Sheen *et al.*, 1999; Rolland *et al.*, 2006). They could also be indicators of stresses in plants and sugar levels are often modulated by environmental stresses (Hanson & Smeekeens, 2009), notably in response to water deficit (Hummel *et al.*, 2010).

Trehalose is a non-reducing sugar, consisting of two units of glucose ( $\alpha$ -D-glucopyranosyl-[1,1]- $\alpha$ -D-glucopyranoside; Fig.1A) and is a ubiquitous sugar that has received a great attention over the last years for its involvement in plant metabolism, physiology and development (for reviews, see Paul *et al.*, 2008; Ponnu *et al.*, 2011). It was identified for the first time in 1832 as a constituent of the ergot fungus of rye. The name trehalose was introduced in 1858: trehalose was found in cocoon of the desert insect *Larinus nidificans* that was called “trehala” by native people (Paul *et al.*, 2008). Trehalose is present in large quantities in bacteria, fungi and invertebrate animals (Elbein *et al.*, 2003) where it plays a dual role as a storage carbohydrate and acts to protect organisms against different stressing conditions (Wingler, 2002). During desiccation, trehalose forms an amorphous glass structure that limits molecular motion and free radical diffusion (Brumfiel, 2004) and thus, protects proteins and membranes from denaturation (Crowe, 2007).

In the plant kingdom, high trehalose levels have been detected (millimolar amounts) in highly desiccation-tolerant “resurrection” plants. In the desert “resurrection” plants *Selaginella lepidophylla* and *Myrothamnus flabellifolia*, trehalose is the main soluble sugar and acts as a protector for both proteins and membranes under desiccation tolerance (Bianchi *et al.*, 1993; Liu *et al.*, 2008). In other plants, trehalose is only present in hardly detectable amounts (low micromolar or less) and seems to mainly act as a signal compound (Grennan, 2007). In plants, trehalose biosynthetic pathway has recently been found and a plethora of genes implicated in trehalose metabolism were identified in *Arabidopsis thaliana* (see references in Leyman *et al.*, 2001; Fig. 1C). Two steps are required to trehalose anabolism under the control of 21 putative genes (Fig. 1C, B). The first step is the formation of the precursor of trehalose from UDP-glucose and glucose-6-phosphate and catalyzed by the enzyme trehalose-6-phosphate synthase (TPS) into trehalose-6-phosphate (T6P). Subsequently, T6P is dephosphorylated into trehalose by trehalose-6-phosphate phosphatase (TPP). The degradation of trehalose includes a unique step leading to the formation of two molecules of



**Fig.2. T6P accumulation has deleterious effects on plant growth (from Paul 2007)**

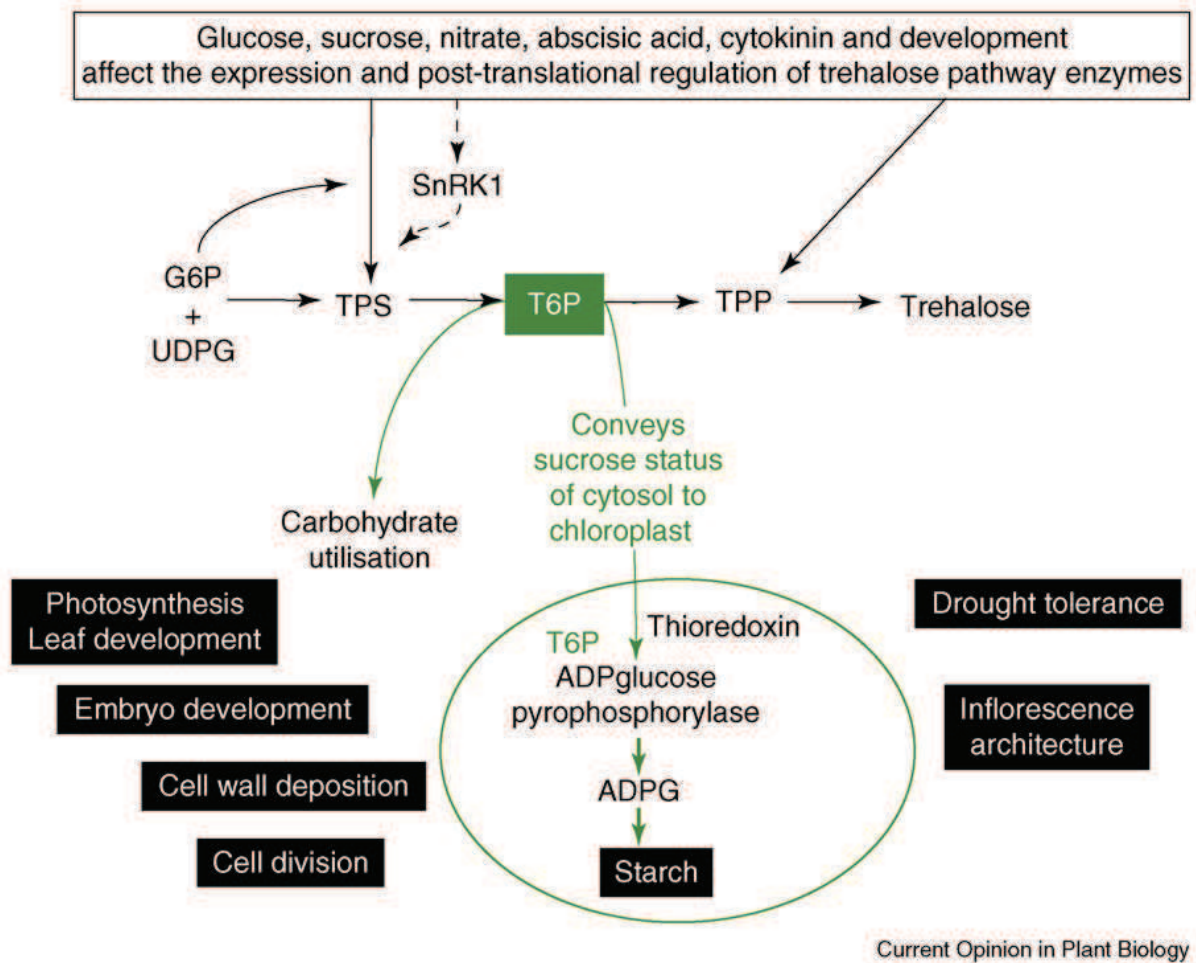
(a) T6P is synthesized from G6P and UDPG, in a pathway that is catalysed by TPS, whereas TPP catalyses the formation of trehalose. (b) The *E. coli* genes *otsA* encoding TPS and *otsB* encoding TPP were transformed into *Arabidopsis* top and bottom plants, respectively. (c,d) Tobacco plants expressing the *E. coli* TPS have small dark leaves (left plants), whereas tobacco plants expressing the *E. coli* TPP have the opposite phenotype (right plants).

glucose under the control of a single gene encoding trehalase enzyme, since no homologue is present in the genome (Tre; Fig. 1C, B). Despite the presence of multiples genes in trehalose anabolism, only few genes present a real significance in Arabidopsis processes. The first experiments revealed that only the Arabidopsis TPS1 is able to complement a yeast mutant deleted in *TPS1* gene (Blazquez *et al.*, 1998). Later others studies revealed that TPS6 (Chary *et al.*, 2008) and also TPS11 (Singh *et al.*, 2011) are also able to do complementation. However, there are no proofs about the enzymatic activity of the other TPS genes (*TPS2-5, TPS7-10*). On the contrary, all *TPP* and trehalase genes encode active enzymes (Muller *et al.*, 2001; Frison *et al.*, 2007; Van Houtte *et al.*, 2013a).

### I.1. The manipulation of trehalose metabolism induces resistance to water stress in plants

Trehalose metabolism has widely been shown to be implicated in plant tolerance to water deficit and trehalose accumulation is observed in various plant species (for review, see Fernandez *et al.*, 2010). Trehalose accumulation can be at least partially caused by transcriptional regulation: analyzes of gene expression by microarray has revealed that trehalose related genes in Arabidopsis are induced during water stress establishment (Iordachescu & Imai, 2008). To create stress tolerant plants, transgenic plants over-producing trehalose have been developed by introducing bacterial or yeast trehalose biosynthetic gene: for instance in Arabidopsis (Karim *et al.*, 2007; Miranda *et al.*, 2007), rice (Garg *et al.*, 2002; Jang *et al.*, 2003) and tobacco (Romero *et al.*, 1997). Resultant transgenic lines accumulating trehalose and T6P display a higher plant survival (Miranda *et al.*, 2007), higher plant biomass (Garg *et al.*, 2002) and a better water status than their wild type controls (Karim *et al.*, 2007). However, in some cases, plants over-producing trehalose display a reduced growth (Fig. 2; Paul, 2007). Karim *et al.*, (2007) discovered that it is necessary to induce an accumulation of trehalose, but not T6P in order to obtain a plant resistant to drought without having deleterious effects of T6P on growth. Moreover, although these transgenic lines present an improvement of water stress tolerance, accumulated trehalose quantities are relatively low. This could be due to (i) a complex regulation of trehalose content or (ii) a possible secretion outside of the cells or (iii) increased trehalase activity that prevents trehalose accumulation (Penna, 2003; Van Houtte *et al.*, 2013a).





**Fig.3. Functional context of T6P in plants (according to Paul 2007).**

A variety of cues and signals including glucose, sucrose, nitrate, abscisic acid, cytokinin and development regulate TPSs and TPPs at the gene level. TPSs are also regulated post-translationally by protein phosphorylation by SnRK1. These mechanisms provide a means for the regulation of T6P levels in different tissues and under different conditions. Whether all pathway genes are involved directly or at all in the regulation of T6P levels remains to be established. T6P regulates carbohydrate metabolism and stress responses that impact on important traits such as photosynthesis, development, cell wall and starch deposition, and drought tolerance.

## I.2. The metabolism of trehalose is implicated in plant developmental processes

In recent years, there has been a growing interest in the role of trehalose metabolism in signaling and plant development, notably by the action of trehalose precursor, T6P as a regulatory molecule (Fig. 3 ; for reviews see, Paul, 2008; Schluempmann *et al.*, 2012). Strong evidences show that trehalose pathway is an essential metabolic signal and is absolutely required for plant development. At first, *AtTPS1* has been shown indispensable for *Arabidopsis* embryo maturation (Eastmond *et al.*, 2002). Loss of *TPS1* gene function leads to growth arrest at the torpedo stage of embryo and is associated with cell division and cell wall structure alterations (Gomez *et al.*, 2006). As *tps1* embryo cannot be saved by the application of exogenous trehalose, the arrest in embryo development is attributed to T6P (Schluempmann *et al.*, 2003). *AtTPS1* is also absolutely essential for a normal vegetative growth and has been recognized as a key gene for regulation of flowering time (van Dijken *et al.*, 2004; Wahl *et al.*, 2013). The *tps1* mutant rescued by inducible expression of *TPS*, shows retarded vegetative growth and are unable to flower (van Dijken *et al.*, 2004). Moreover, recently a study that used a weak allele of *tps1* obtained with microRNA, confirmed the crucial role of T6P in the regulation of flowering (Wahl *et al.*, 2013). Trehalose metabolism is also implicated in inflorescence architecture. This is illustrated by the mutant *ramosa3* of maize that present a lesion in the gene encoding a functional TPP enzyme and an increase in inflorescence branching (Sato-Nagasawa *et al.*, 2006). Moreover, recent dosages in the inflorescence primordia of *ramosa3* and wild maize show that trehalose content or trehalose/T6P ratio are more implicated in inflorescence development than T6P content (Carillo *et al.*, 2013). Although T6P is essential for embryogenesis, flowering and normal vegetative growth, its accumulation may induce deleterious effects. The comparison between transgenic over-expressing *TPS* gene (more T6P than in wild plant) or over-expressing *TPP* gene (less T6P than in wild plants) indicates that T6P accumulation provokes growth inhibition in *A.thaliana* (Fig. 2; Schluempmann *et al.*, 2004). As a hormone, T6P content is finely regulated in plants and its effects seem to be dose-dependent.

### I.3. Trehalose metabolism affects sugar metabolism, carbohydrate utilization and plant physiology

In addition to the involvement of trehalose metabolism in plant development, this metabolite has also significant effects on carbohydrate utilization and plant physiology. T6P and sucrose are closely related (Lunn *et al.*, 2006). Sucrose feeding leads to a rapid and large increase in T6P content. For example, T6P content increases 40 fold when C-starved plants were grown on sucrose supplemented medium. Moreover, the level of T6P follows the sucrose level during diurnal cycle. The correlation between sucrose and T6P suggests that T6P may act as a signal of sucrose status (Lunn *et al.*, 2006). Under high sucrose condition, T6P presents multiple roles in starch metabolism. One of the most documented is the T6P effect on starch accumulation. Indeed, starch accumulation occurs in response to trehalose feeding, a condition in which an increased in T6P content is observed (Wingler *et al.*, 2000). Moreover, T6P controls the enzyme of starch synthesis ADPglucose pyrophosphorylase (AGPase) by a thioredoxin-dependent redox activation (Fig. 3 ; Kolbe *et al.*, 2005; Lunn *et al.*, 2006). It has been shown that T6P also participates to a feedback inhibition of starch degradation (Martins *et al.*, 2013). Recently, it was shown that T6P interacts with SnRK1, a central transcriptional regulator that responds to carbon status and energy supply (Hardie, 2007; Delatte *et al.*, 2011). SnRK1 regulates over 1000 genes involved in biosynthetic, growth, and stress responses (Baena-González *et al.*, 2007). T6P accumulated in high sugar conditions, inhibits SnRK1 and thus induces genes involved in growth processes and inhibits those involved in stress responses (Nunes *et al.*, 2013).

Trehalose metabolism might also participate in sugar sensing through trehalase activity (Lunn, 2007). In *A. thaliana*, trehalase is a membrane protein, with the active hydrolase domain located on the apoplastic side of the plasma membrane, a strategic location in signaling (Frison *et al.*, 2007).

A remarkable effect on plant physiology is the enhancement of photosynthetic capacity by trehalose metabolism (Paul *et al.*, 2001; Pellny *et al.*, 2004). Transgenic plants expressing *E. coli* trehalose related genes display a significant increase in photosynthetic capacity per unit leaf area, associated with alterations in Rubisco activity by T6P content (Pellny *et al.*, 2004). Moreover, *TPS6* gene was found implicated in trichome branching, shape of epidermal cells and general plant architecture (Chary *et al.*, 2008).

#### I.4. Trehalose metabolism is implicated in the interaction between plants and microorganisms

Over the last years, trehalose metabolism has generated enthusiasm and played an important role in plant biology researches. However, despite rapid knowledge acquired over the years, few investigations have been performed on the role of trehalose on plant-microbes interactions. A few pieces of evidence indicate that trehalose metabolism is implicated in plant-microorganisms interaction, notably during symbiosis or plant-pathogen interactions.

Trehalose plays a role in the establishment of plant/ectomycorrhizal symbiosis (Wiemken, 2007) but also in plant infection by pathogen illustrated by a decrease of pathogenicity of *Magnaporthe grisea* observed in the deletion *tps1* mutant (Foster *et al.*, 2003). Moreover, trehalose has a particular role in symbiosis between rhizobacteria and *Leguminosae* (Fernandez *et al.*, 2010). High quantity of trehalose is found in nodules and bacteroids suggesting that trehalose is important to nodule functioning. *Bradyrhizobium japonicum* mutated in trehalose synthesis related genes produces fewer mature nodules on soybean roots than inoculation by wild strain (Sugawara *et al.*, 2010). Trehalose accumulated in nodule and cytoplasm of host plant cells can be beneficial to plants: trehalose concentration correlates with improvement of plant host tolerance to stress (Farias-Rodriguez *et al.*, 1998). Moreover, inoculation of common bean (*Phaseolus vulgaris*) by a transgenic of *Rhizobium elti*, a high trehalose producer, lead to the development of more nodules and to increased biomass compared to host plants inoculated by the wilt-type strain (Lopez *et al.*, 2008; Suarez *et al.*, 2008). Recently, it was also found that plant trehalose metabolism can also be beneficial for bacteria during the symbiosis. A transgenic common bean with trehalase gene inhibited by RNAi displays an enhancement in bacteroid numbers in nodule (Barraza *et al.*, 2013). The trehalose secreted into soil by plants could represent a nutrient for microorganisms and seems to be important for plant/microorganism interaction (Fernandez *et al.*, 2012).

Plant trehalase activity may also have a particularly important role in plant/microorganisms interaction. Trehalase was also found with a very high activity in root nodule of legumes (Muller *et al.*, 1995). Trehalase is implicated in plant responses to pathogens. For instance, trehalase gene and trehalase activity were found to be strongly induced in *Arabidopsis* roots infected by the trehalose-producing pathogen *Plasmodiophora brassicae* (Brodmann *et al.*, 2002). This suggests that trehalase might be acting in plant

defense processes by counteracting trehalose accumulation in infected tissues (Brodmann *et al.*, 2002).

Finally, few studies describe the implication of trehalose metabolism in mutualistic interaction with plant growth promoting rhizobacteria (PGPR). Recently, it has been shown that a natural PGPR, *Burkholderia phytofirmans* induces an accumulation of T6P and trehalose in the stem and leaves of grapevine (Fernandez *et al.*, 2012). Inoculation by a transgenic over-producing trehalose *Azospirillum brasilense* allows drought resistance in maize. This result suggests that trehalose produced by a PGPR can also have a beneficial effect on plant resistance to stress (Rodriguez-Salazar *et al.*, 2009).

All these examples show that trehalose metabolism is crucial for the interaction between plant and microorganisms, but we are still far from a thorough understanding of underlying mechanisms, especially under water stress.

The main goal of the Bruno Touraine team was to understand, at the molecular level, how the inoculation by the PGPR *Phyllobacterium brassicacearum* STM196 modifies nutrition, development and growth of the plant *Arabidopsis thaliana*. To find new mechanisms, a transcriptome and a metabolome were performed on *Arabidopsis* cultivated *in vitro* and inoculated or not by STM196 (Delteil *et al.*, *in prep.*). The analysis of the metabolome reveals that T6P and trehalose were over-accumulated in *Arabidopsis* 7 days after inoculation. In parallel, the transcriptome revealed that *TPS11* transcripts were also accumulated in *Arabidopsis* treated with STM196. These results suggest that STM196 promotes T6P and trehalose accumulation through the transcriptional activation of *TPS11*. Moreover, kinetic analysis of trehalose and T6P contents and level of accumulation of *TPS* transcripts also confirmed the promotion of trehalose and T6P accumulation in *Arabidopsis* 6 days after STM196-inoculation (Delteil *et al.*, *in prep.*).

In this study, we tested trehalose and T6P as functional molecules in *A. thaliana* responses to inoculation by the STM196 strain under stressing conditions. Experiments performed in soil that showed inoculation by STM196 strain induces an increase of *A. thaliana* resistance to moderate water deficit through phenological and physiological changes (manuscript 2; chapitre 3) and improves plant survival under severe water stress (manuscript 3; chapitre 4). We developed a functional biology approach to decipher trehalose implication in developmental and physiological changes induced by STM196 in plants. We selected three mutants affected in gene encoding trehalase. Two contrasted mutants are affected in endogenous gene coding *A. thaliana* trehalase (AtTRE1) and present a knock-out and over-

expression, respectively. Another over-expressing mutant carrying the trehalase gene of *Escherichia coli* (TreF) was used. The high-throughput plant phenotyping platform PHENOPSIS (Granier *et al.*, 2006) was used to apply two different water deficit scenarios on plants in order to examine the mechanisms by which inoculation can help plants dealing with water stress through trehalose modifications. In the first scenario, a moderate water deficit was applied during the whole plant life cycle whereas in the second scenario a severe water stress causing plant death was imposed by stopping irrigation and followed by a recovery phase.

We design this experiment to improve the knowledge about (i) the effects of trehalase alterations in plant metabolism and growth (ii) the implication of trehalase in plant resistance to water stress, and (iii) to have a functional proof of the involvement of trehalose metabolism in plant responses to STM196 inoculation under water stress.

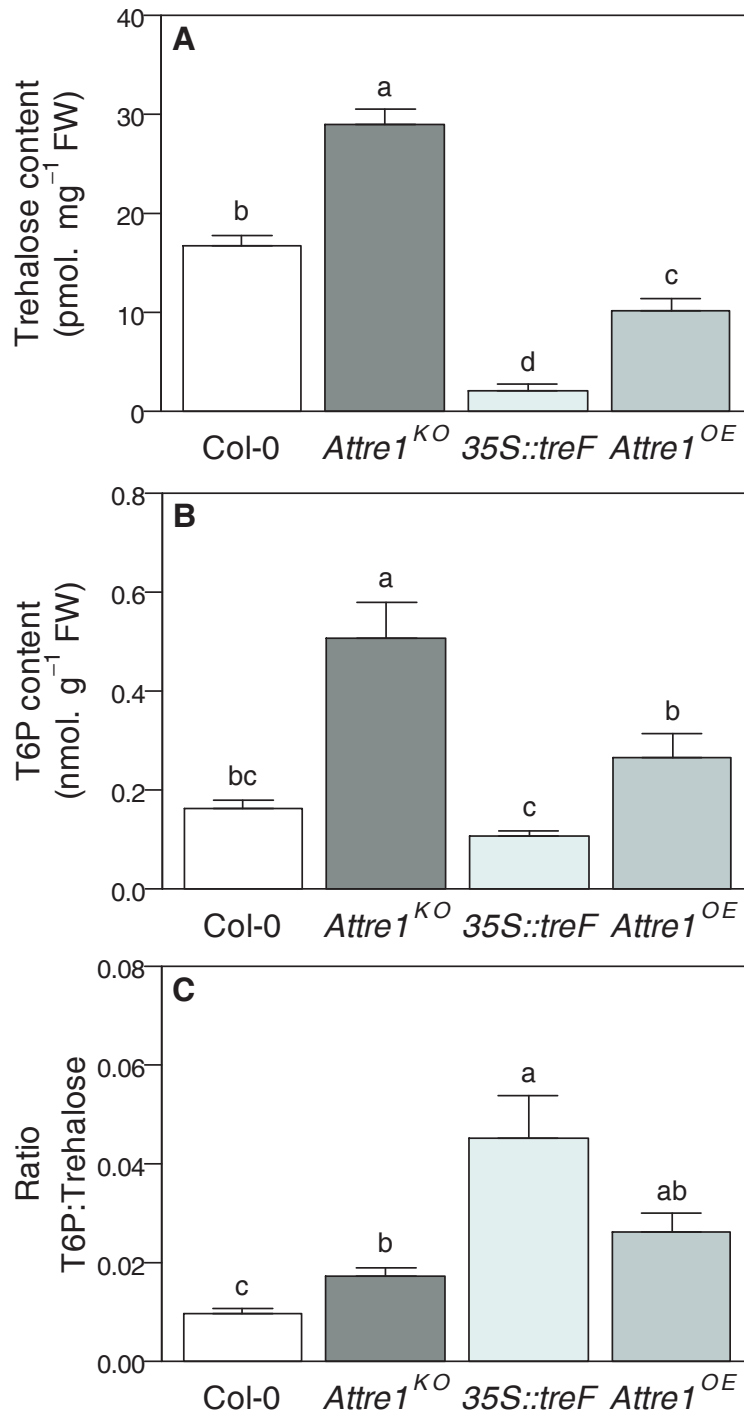
## II. Results and Discussion

### II.1. Trehalase-modified mutants display strong modifications in their metabolism and growth that contrast with findings in *TPP-TPS* mutants

Trehalase is the only specific enzyme known to be responsible for the degradation of trehalose. However, very few studies have focused on the effect of altering trehalase activity whereas the literature is abundant on the implication of trehalose biosynthesis genes on plant growth, notably the *TPS1* gene (for review, see Ponnu *et al.*, 2011). To assess the influence of trehalase on plant development and metabolism, three *Arabidopsis* mutants with altered trehalase gene (*TRE1*) expression were used in this study.

#### II.1.1. Transgenic lines with modified expression of *TRE1* are affected in the level of trehalose, T6P and T6P/trehalose ratio

As expected, *Attre1<sup>KO</sup>* lines (SALK\_147073 from the SALK collection; Alonso *et al.*, 2003) a knockout mutant in endogenous gene, showed a 1.7-fold increase in trehalose content in leaves compared to the wild-type Col-0 (Fig. 4A;  $P < 0.001$ ). It is generally assumed that trehalase is the key enzyme to improve trehalose accumulation in plants (Penna, 2003). However, the accumulation in *Attre1<sup>KO</sup>* lines was not considerable compared to transgenic TPP and TPS lines. In *Arabidopsis*, the trehalose content of transgenic lines overexpressing

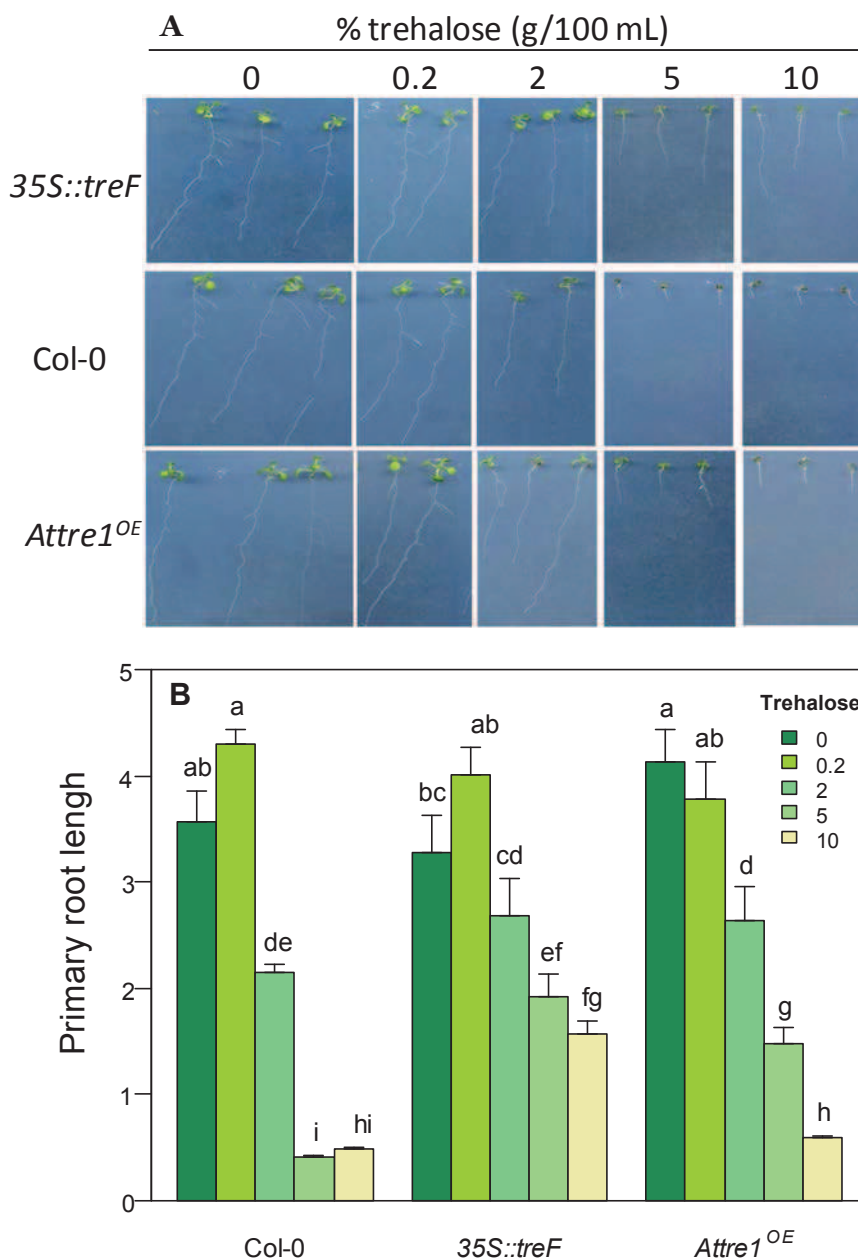


**Fig.4. Trehalose metabolism was affected in *AtTRE1*-modified mutants.** (A) Trehalose, (B) T6P content and (C) ratio T6P: trehalose in leaves of plants harvested at bolting stage. Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ) between lines: Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>. Trehalose and T6P measurements were executed as described in Lunn *et al.* (2006).

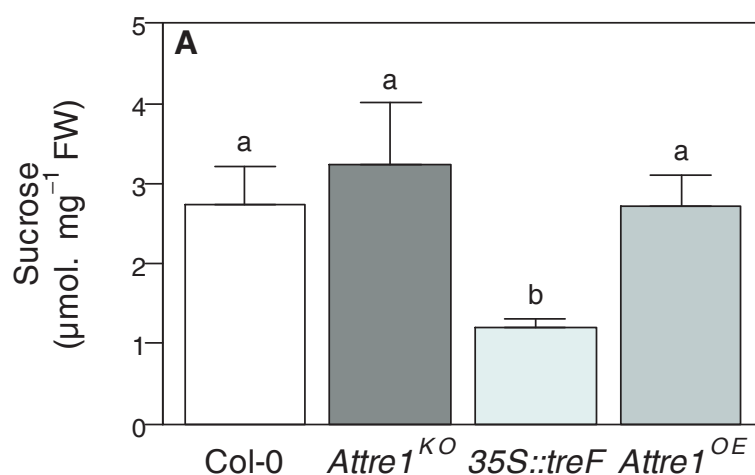
the bifunctional *TPS1-TPS2* gene could be significantly increased by 1.1 to 5 times compared to wild-types (Miranda *et al.*, 2007). In rice, Garg *et al.* (2002) has shown transgenic lines expressing the *E. coli* TPS-TPP that had a trehalose content 3 to 8 times that of the non-transgenic plants. Inhibiting trehalase is probably not the best way to induce a large trehalose accumulation in Arabidopsis. Moreover, it has been shown in Arabidopsis that inhibition by a specific trehalase inhibitor Validamycin A induces an increase of trehalose in tissues only with exogenous trehalose application (Muller *et al.*, 2001). This difficulty to accumulate trehalose by trehalase breakdown can be explained by negative feedback of trehalose on its biosynthesis but also by activity of another glycosidase, such as broad-specificity acid  $\alpha$ -glucosidase reported in some legume species (Van Houtte *et al.*, 2013b). Another hypothesis to explain the difficulty to over-accumulate trehalose in plants is that trehalose could be secreted outside plant cells (Fernandez *et al.*, 2012).

Two contrasted overexpressing lines were also used. The *Attre1<sup>OE</sup>* line (SAIL\_25C12 from the SAIL collection; Sessions *et al.*, 2002) overexpressed the endogenous gene which encodes AtTRE1 protein should be targeted to plasmalemma with the active site facing to apoplast (Frison *et al.*, 2007) and the *35S::treF* (provided by Dr. J. E. Lunn; MPI-MP; Golm; Carillo *et al.*, 2013) presented a constitutive expression of *E. coli* cytosolic trehalase gene (*TreF*). Overexpression of trehalase gene in *35S::treF* and *Attre1<sup>OE</sup>* mutants induced a 8.4-fold and a 1.6-fold decrease in trehalose content, respectively (Fig. 4A, both  $P < 0.001$ ). Regarding the reduction of trehalose level, in *A. thaliana* the cytosolic TREF trehalase was more efficient than the apoplastic endogenous TRE1 trehalase. Modifications in expression of *AtTRE1* induced changes in trehalose but also in T6P content. Leaf T6P content was significantly increased in *Attre1<sup>KO</sup>* ( $P < 0.001$ ) and had similar levels in overexpressing lines compared to wild-type (Fig. 4B). The increase in trehalose content was correlated with an increase in T6P content (Fig. S1). However, the work of Brodmann (2006) suggests that the large accumulation of intracellular trehalose induces a negative feedback of TPPs leading to T6P accumulation. Some authors also suggest that the T6P/trehalose ratio may have an important role in the regulation of carbohydrate metabolism or development (Carillo *et al.*, 2013). Interestingly, the ratio between T6P and trehalose was also increased in the three transgenic lines (Fig. 4C;  $P < 0.05$ ). This showed that changes in *TRE* expression induced imbalances between trehalose and its precursor. This result is in accordance with studies that were performed on over-expressing *TRE* lines, *Attre1-3<sup>OE</sup>* and *35S::treF* displaying a higher T6P/trehalose ratio than wild type Col-0 (Van Houtte *et al.*, 2013b).





**Fig.5. Transgenic plants over-expressing *TRE1* displayed a better resistance to exogenous trehalose *in vitro*.** (A) Plant phenotypes and (B) primary root length of plants after 7 days of growth from 0 to 10% of trehalose content in plant medium. Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ) between lines: Col-0, wild-type and the over-expressing lines *35S::treF* and *Attrel1<sup>OE</sup>*.



**Fig.6. The relationship between sucrose and T6P in transgenic lines was complex.** Sucrose content in leaves of plants harvested at bolting stage. Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ) between lines: Col-0, wild-type, *Attrel1<sup>KO</sup>* the knockout mutant and the over-expressing lines *35S::treF* and *Attrel1<sup>OE</sup>*. Plants were harvested at the bolting stage, in the middle of the day, and immediately frozen in liquid nitrogen. Sucrose content was determined by enzymatic assay as in Gibon *et al.* (2004). 126

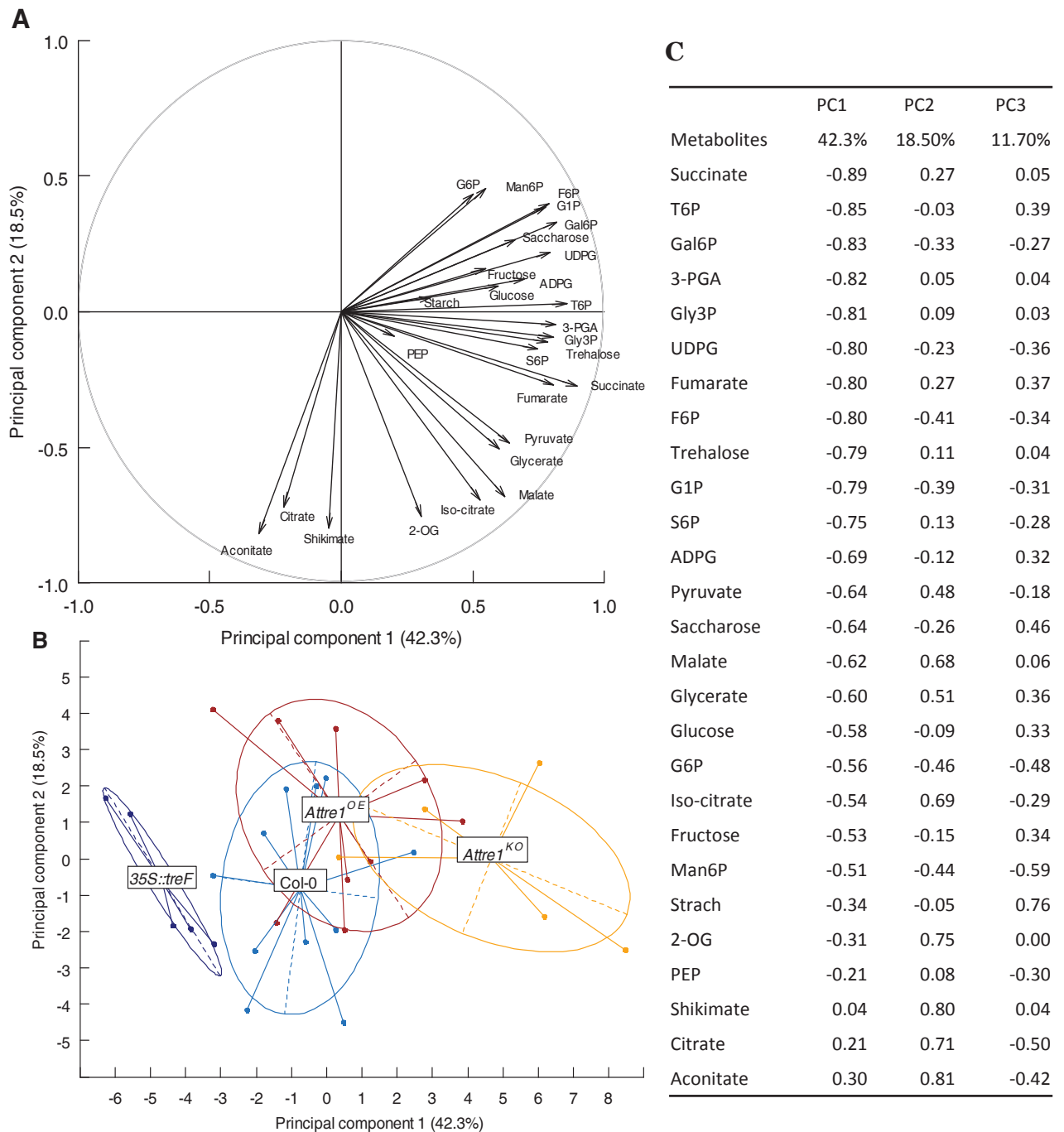
### II.1.2. Transgenic plants over-expressing *TRE1* display a better resistance to exogenous trehalose in vitro

The difference of efficiency of trehalase activity between *35S::treF* and *Attre1<sup>OE</sup>* was assessed by *in vitro* analysis of root growth in presence of exogenous trehalose. It has been shown that adding exogenous trehalose at high concentration to the plant culture media leads to abnormal and reduced growth (Ramon *et al.*, 2007). Primary root length of these two overexpressing *TRE* lines and wild-type seedlings was measured after 7 days of growth from 0 to 10% of trehalose content in plant medium. At low trehalose exogenous concentration (from 0 to 0.2%), transgenic plants displayed a normal phenotype with a primary root length identical to wild-type plants (Fig. 5). In the presence of high trehalose exogenous concentration (from 2 to 10%), all seedlings failed to growth and develop rosettes leaves (Fig. 5A). Primary root length was significantly decreased under high trehalose concentrations, but *35S::treF* and *Attre1<sup>OE</sup>* displayed a smaller reduction in primary root length compared to Col-0 (Fig 5B ;  $P < 0.05$ ). This result suggests that overexpressing lines were more tolerant to the external supply of trehalose. Moreover, *35S::treF* lines displayed a better root growth than *Attre1<sup>OE</sup>* under 10% of trehalose supply (Fig. 5B ;  $P < 0.05$ ). This result is consistent with a higher trehalase activity in *35S::treF* that could confer the strong differences observed with the wild type and *Attre1<sup>OE</sup>* (Van Houtte *et al.*, 2013b). Other over-expressing *TRE* lines were tested in Van Houtte *et al.* (2013b) and also display a better resistance to high exogenous trehalose than Col-0, related to high trehalase activity. They also tested the *Attre1<sup>KO</sup>* lines that present more pronounced growth inhibition than Col-0 under high trehalose concentration.

### II.1.3. Transgenic lines with modified expression of *TRE1* are affected in carbon metabolism

We have already mentioned that fluctuations in the level of T6P are known to be parallel to the level of sucrose (Lunn, 2007). Here, in transgenic lines the relationship between sucrose and T6P was more complex (Fig. 4, 6). The *35S::treF* line that had a large reduction of T6P content had much reduced sucrose levels, while *Attre1<sup>KO</sup>* line, that exhibited a greater T6P content than Col-0, did not presented any change in sucrose levels. Unsurprisingly, no significant difference was found in T6P and sucrose contents in *Attre1<sup>OE</sup>* lines (Fig. 4, 6).

In the same way, it is widely assumed that over-accumulating T6P plants exhibit an increase in starch content (Kolbe *et al.*, 2005; Martins *et al.*, 2013). In our case, transgenic



**Fig.7. Transgenic lines with modified expression of TRE1 are affected in carbon metabolism.** Principal component analysis (PCA) on multiple metabolites measured on plants harvested at bolting stage. The first two axes are shown which account for 61% of the total inertia. **(A)** Projection of the metabolites, **(B)** projection of individual plants and **(C)** loadings of the variables on the three firsts principal components. Col-0, wild-type, *Attre1<sup>KO</sup>* the knockout mutant and the over-expressing lines *35S::treF* and *Attre1<sup>OE</sup>*. Metabolite measurements were executed as described by Lunn *et al.*, 2006

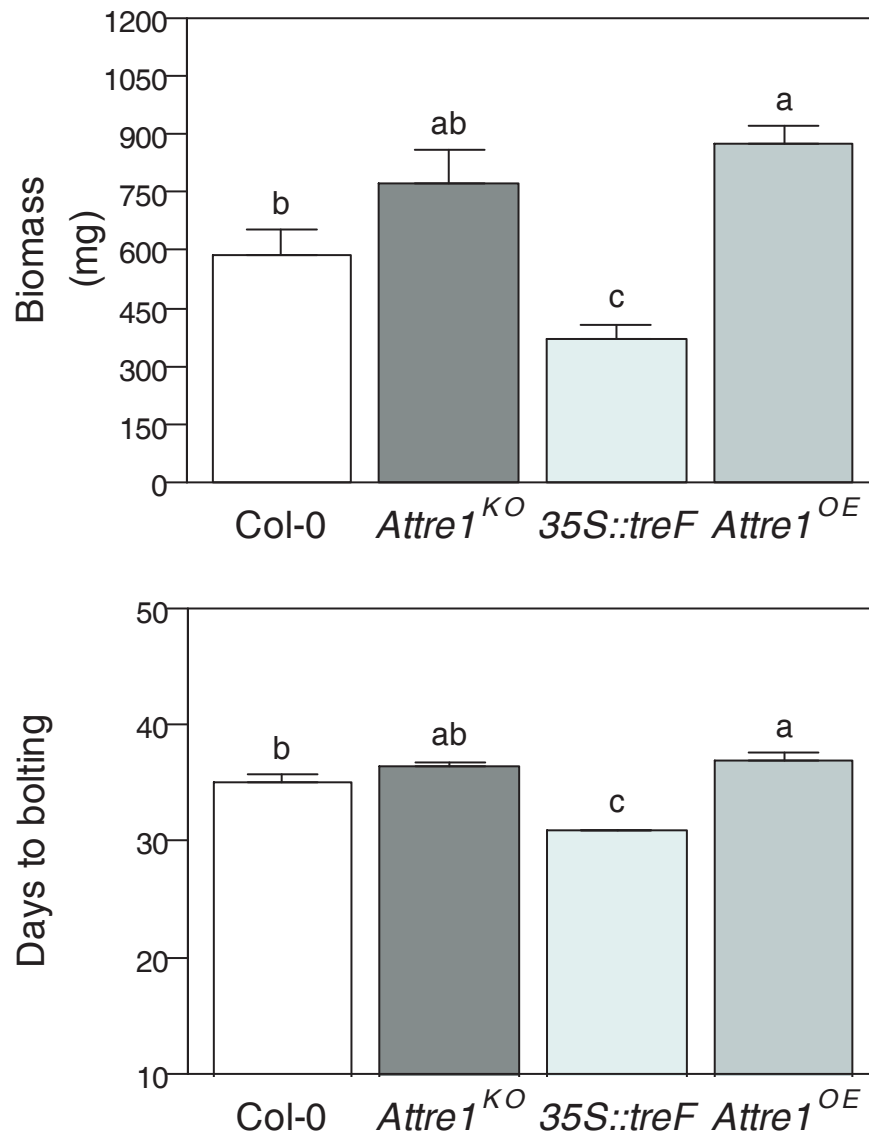
modifications of trehalase did not produce any modification in starch content (data not shown).

Transgenic plants also differed from wild-type in many other metabolites. A principal component analysis on 27 metabolites (Fig. 7) showed that the first and second principal components (PCs) explained 42.3% and 18.5% of the total variance, respectively. PC1 was mainly explained by trehalose, T6P and succinate and PC2 by shikimate, citrate and aconitate (Fig. 7A and C). Projection of individuals revealed significant effects of the genotype with a strong effect of *35S::treF* and *Attrel<sup>KO</sup>* lines on PC1 in an opposite way (Fig. 7B;  $P < 0.001$  ANOVA on PC coordinates). *Attrel<sup>KO</sup>* and *35S::treF* differed from Col-0 on PC1 by drastic changes in trehalose and T6P but also in succinate, 3-PGA, Gly3P and UDPG (Fig. 7 and S2). The major effects were represented by an increase of metabolite contents in *Attrel<sup>KO</sup>* and a decrease in *35S::treF* (Fig. S2;  $P < 0.05$ ). This result illustrated that *Attrel<sup>KO</sup>* and *35S::treF* mutants that had greatly opposite effects on trehalose/T6P levels, had also opposite effects on other metabolites. In contrast, on PC2, the projection individuals of overexpressing *Attrel<sup>OE</sup>* lines revealed no significant difference to Col-0 (Fig. 7; ANOVA on PC coordinates). The great difference between the metabolome of these two overexpressing lines could be due to differences in trehalase activity and location. Taken together, our results have shown a large imbalance in the carbon metabolism of *35S::treF* and *Attrel<sup>KO</sup>* lines. The overexpressing *Attrel<sup>OE</sup>* lines had less contrasted difference with wild-type plants.

Trehalase-modified mutants conferred contrasted behavior compared to findings in the literature on trehalose/T6P implication. All of these results also suggest that trehalase activity is crucial to maintain the correlations between T6P and sucrose/starch, and seems to be implicated in a complex and obscure regulation of plant carbon metabolism and/or signaling.

#### II.1.4. Transgenic lines with modified expression of TRE are affected on growth and flowering time

Trehalose/T6P metabolism was strongly implicated in plant growth and T6P has recently been shown to regulate flowering time in *A. thaliana* (Wahl *et al.*, 2013). Here, we showed that only overexpression of trehalase induced a dramatic effect on plant growth (Fig. 8). A large reduction of biomass at the apparition of flower buds (bolting) was observed in *35S::treF*, while *Attrel<sup>OE</sup>* lines displayed a great increase (Fig. 8A). Since growth and flowering time are closely related, it is not surprising to observe an early flowering in *35S::treF* and a delayed flowering in *Attrel<sup>OE</sup>* lines. However, these mutants showed a



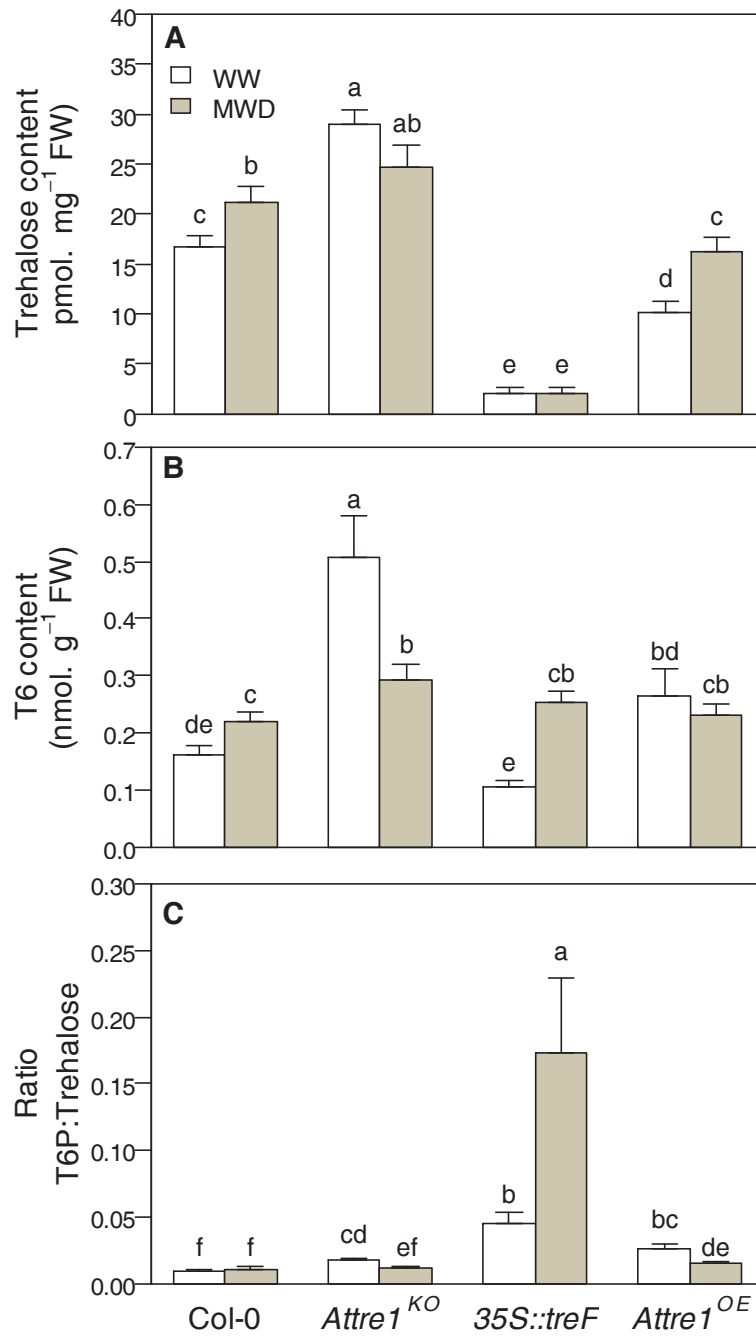
**Fig.8. Transgenic lines with modified expression of *TRE1* are affected on growth and flowering time.** (A) Biomass of plants harvested at bolting stage and (B) days to reach at bolting stage. Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ) between lines: Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>.

decoupling between T6P content and flowering time and did not reflect the implication of T6P findings in *tps1* mutants. The knock-down *TPS1* expression lead to a significant 25 to 30% reduction in T6P levels and a delay in flowering time by more than 20 leaves (Wahl *et al.*, 2013). A few pieces of evidence indicate that trehalase could be important during plant development. In *A. thaliana*, *AtTRE* transcripts are particularly abundant in flowers and developing seeds/siliques (Schmid *et al.*, 2005) suggesting that trehalase could regulate T6P and trehalose content in floral meristem and influence flowering time.

At first sight, overexpression of trehalase in *A. thaliana* had little effect on phenotype in contrast to the strong phenotypes produced by overexpressing TPS and TPP genes (Schluepmann *et al.*, 2003). However, we showed that altered trehalase activity induced dramatic pleiotropic effects, such as growth trajectory changes and altered carbon metabolism. Although it is clear that trehalase has a strong implication in trehalose/T6P regulation, little is known about the exact mechanism of action on plant development.

## II.2. Trehalase-modified lines exhibit opposite responses under moderate and severe water stress

To understand possible implications of trehalase in plant responses to water deficit, the three modified lines and wild-type Col-0 were submitted to two contrasted soil water deficits. Under well-watered conditions (WW), soil relative water content ( $RWC_{soil}$ ) was maintained at 35%  $g\ H_2O\ g^{-1}$  dry soil (corresponding to a soil water potential of  $-0.07\ MPa$ ). A  $RWC_{soil}$  lower than 30% began to affect *A. thaliana* development (Granier *et al.*, 2006). In the first stress scenario, a moderate water deficit (MWD) was applied during the whole plant life cycle by stopping irrigation from two first leaves emerged and by maintaining soil humidity at 20%  $g\ H_2O\ g^{-1}$  dry soil (corresponding to a soil water potential of  $-0.28\ MPa$ ) until the emergence of flower buds (i.e. bolting). All measured traits in plants were performed at bolting. In the second stress scenario, a severe water deficit (SWD) was applied by progressive decrease of  $RWC_{soil}$  to 6%  $g\ H_2O\ g^{-1}$  dry soil by stopping of watering, then irrigation was restarted to reach the control soil water content (WW; 35%  $g\ H_2O\ g^{-1}$  dry) by daily adding a constant volume of a modified one-tenth-strength Hoagland solution (Hoagland & Arnon, 1950).



**Fig.9. Trehalose metabolism of *AtTRE1*-modified mutants under moderate water deficit.** (A) Trehalose, (B) T6P content and (C) ratio T6P: trehalose in leaves of plants harvested at bolting stage under well watered condition (WW) and moderate water deficit (MWD). Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ). Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>. Trehalose and T6P measurements were executed as described in Lunn *et al.* (2006).

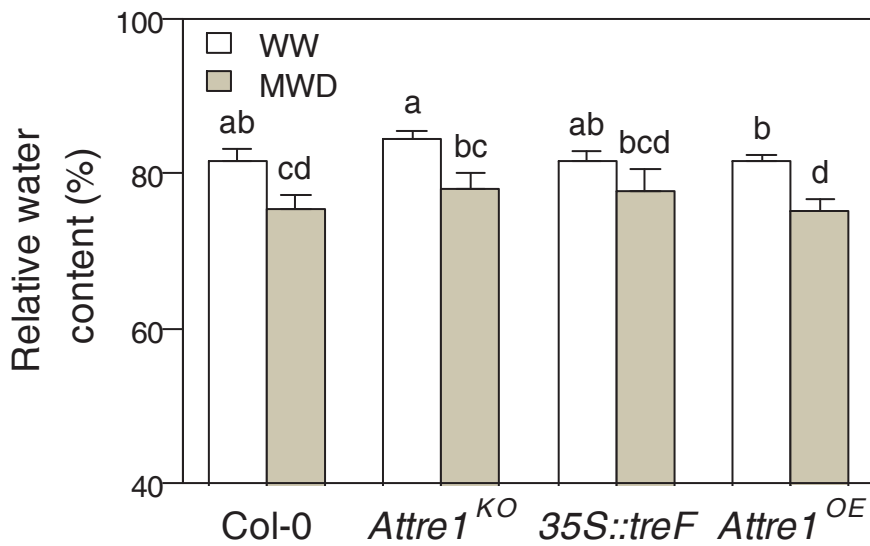
II.2.1. Transgenic lines present modification in trehalose content but not in T6P content under MWD.

The analysis of trehalose metabolism revealed that MWD induced a significant increase in trehalose and T6P content in Col-0 ( $P < 0.05$ ), without changing in the T6P: trehalose ratio (Fig. 9). The increase of trehalose content following a drought stress has already been reported in plants (e.g., Farias-Rodriguez *et al.*, 1998; Garg *et al.*, 2002; El-Bashiti *et al.*, 2005; Iordachescu & Imai, 2008).

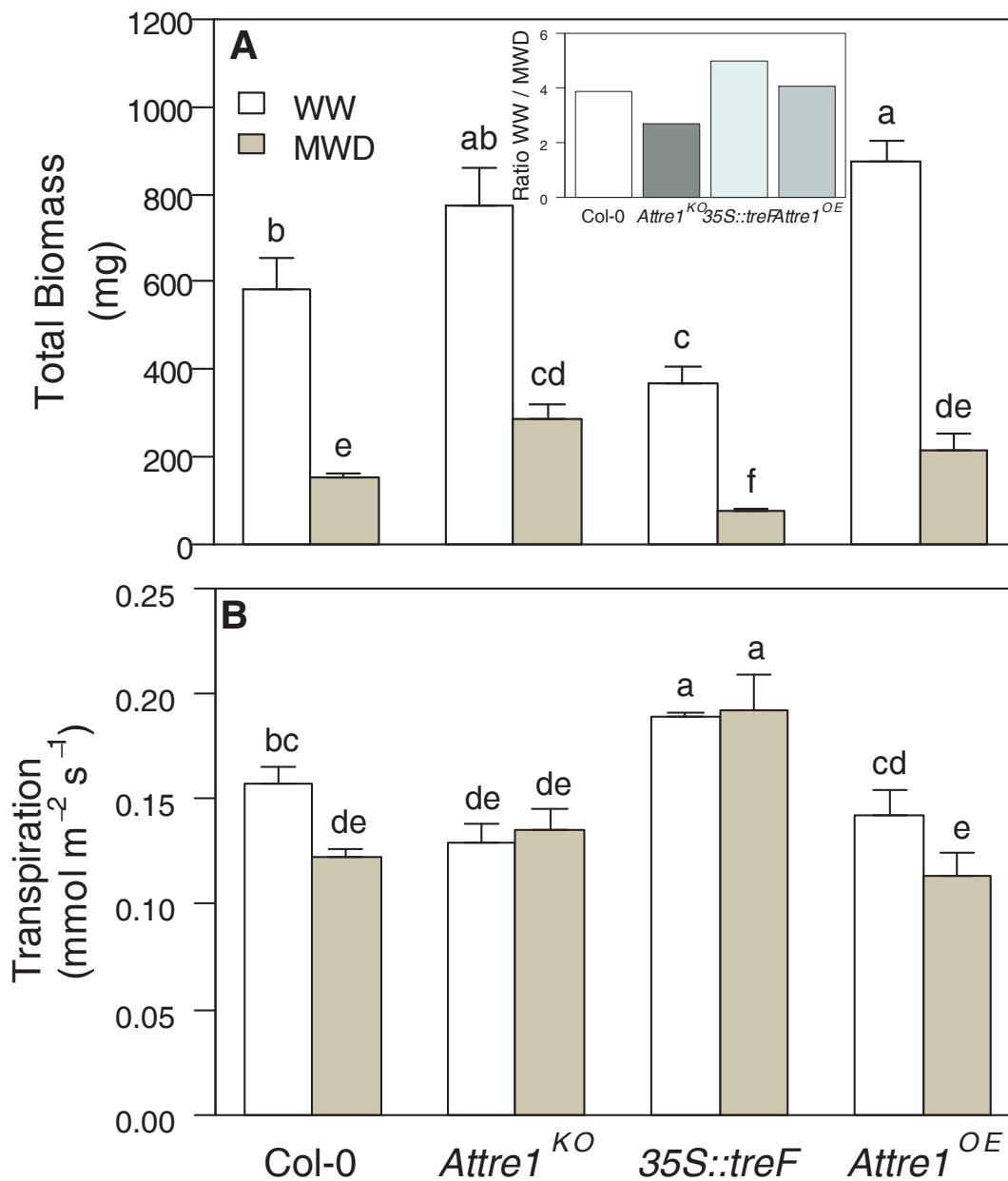
Trehalase-modified lines displayed contrasted responses compared to Col-0 and each line presented a different response to MWD. *Attrel<sup>OE</sup>* lines had a similar response to MWD than Col-0 with an increase in trehalose content ( $P < 0.05$ ; Fig. 9A). However, trehalose content in *Attrel<sup>OE</sup>* was lower than in Col-0 in both WW and MWD conditions. Moreover, *Attrel<sup>OE</sup>* did not present any modification in T6P content with similar level of T6P than Col-0 whatever the soil condition (Fig. 9B). This indicated that the mutation in *Attrel<sup>OE</sup>* had an effect only on trehalose content under MWD. By contrast, modification in trehalose content by MWD was no longer observed in *35S::treF* (Fig. 9A). T6P content in *35S::treF* was 2.4-fold increased, leading to an increase in T6P: trehalose ratio in response to MWD (both  $P < 0.01$ ) but T6P content reached a similar level than Col-0 (Fig. 9B,C). Thus, the over-expression of *TreF* in Arabidopsis allowed maintaining a low trehalose content regardless of the soil conditions and more surprisingly under MWD where it is usually observed an increase in trehalose. *Attrel<sup>KO</sup>* had the most atypical response to MWD. Trehalose content in *Attrel<sup>KO</sup>* was higher than in Col-0 in both WW and MWD conditions. Surprisingly, *Attrel<sup>KO</sup>* had a great reduction of T6P content ( $P < 0.01$ ) that tended to recover a similar level than Col-0 under MWD (Fig 9B).

These results demonstrated that affecting the level of trehalase expression is a good way to modify trehalose content in plant under MWD. Indeed *35S::treF* and *Attrel<sup>OE</sup>* had lower and *Attrel<sup>KO</sup>* had higher trehalose content than Col-0 regardless soil conditions. On the opposite, transgenic trehalase lines are inappropriate to modify T6P content compared to Col-0 under MWD. Thus, trehalase lines are good tools allowing the study of trehalose effect on resistance to MWD without modification in T6P content.





**Fig.10. Water status of *TRE1*-modified mutants under moderate water deficit.** Relative water content in leaves of plants harvested at the bolting stage under well watered condition (WW) and moderate water deficit (MWD). Rosettes were cut and immediately weighed to determine above-ground vegetative fresh mass (FM).



**Fig.11. Trehalase implication in plant responses to moderate water deficit.** (A) Total biomass and (B) transpiration rate of plants harvested at bolting stage under well watered condition (WW) and moderate water deficit (MWD). Insert in (A) represent the ratio of biomass under WW and MWD. Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ).

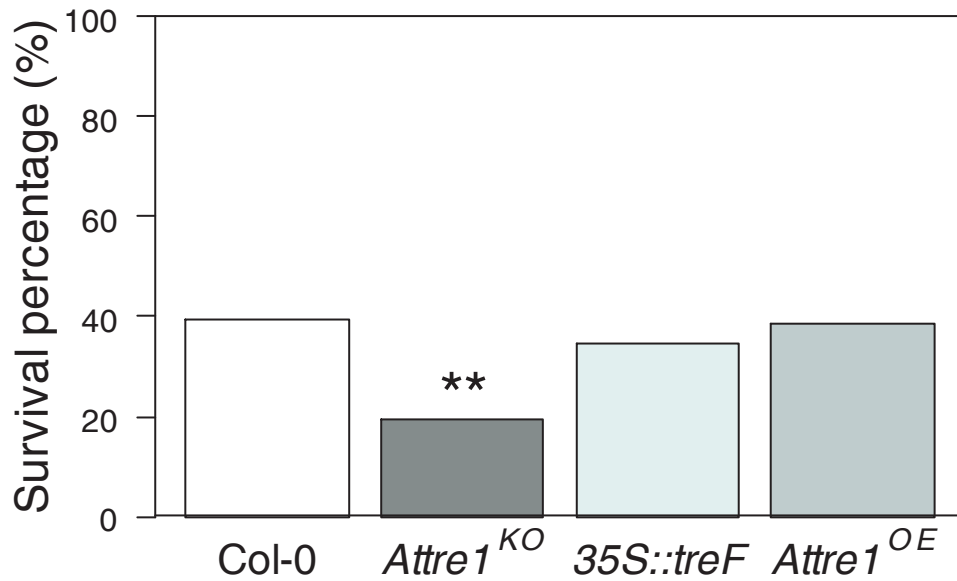
### II.2.2. Trehalose content is positively correlated to resistance to MWD via modifications in transpiration rate

During water stress, plants develop strategies to maximize water gain and diminish water losses. Here, MWD induced a slight decrease of relative leaf water content in all plants from 84.5-81.7% to 75.1-77.9% (Fig. 10). The three trehalase modified lines did not present any difference in their leaf relative water content under both WW and MWD conditions (Fig. 10). Thus, modifications in trehalose content do not affect relative water content in plants regardless of the soil condition.

All plants were significantly affected in their biomass production under MWD (Fig. 11A). The water stress induced a 3.9-fold decrease in biomass of wild-type Col-0 (i.e. WW/MWD ratio; insert in Fig. 11A), and also a significant reduction of transpiration rate (Fig. 11B;  $P < 0.01$ ). However, as expected, *Attre1<sup>KO</sup>* lines, which displayed a large increase in trehalose (Fig. 10), exhibited a higher biomass with a lower WW/MWD biomass ratio (2.7) compared to Col-0 under MWD (Fig. 11A;  $P < 0.01$ ). Under both WW and MWD, *Attre1<sup>KO</sup>* lines had also a lower transpiration rate than Col-0 and reached a similar transpiration rate to wild-type under stress (Fig. 11B). Surprisingly, transpiration rate of *Attre1<sup>KO</sup>* lines was not significantly affected by MWD. These results suggest that the knockout mutant could have a better regulation of water losses and was more resistant to MWD than Col-0.

Although it is largely assumed that trehalose accumulation in plants confers resistance to water deficit, the opposite effect, i.e. less trehalose in plants, has not been related to an exacerbated susceptibility to stress. This could be tested using of over-expressing trehalase mutants. Indeed, *35S::treF* line which had a great decrease in trehalose (Fig. 10), presents a higher WW/MWD biomass ratio (insert in Fig. 11A) and thus, a higher sensitivity to MWD. Moreover, transpiration rate of *35S::treF* was higher than Col-0 regardless soil condition (Fig. 11B;  $P < 0.05$ ). As in *Attre1<sup>KO</sup>*, *35S::treF* lines were not affected in transpiration rate under MWD (Fig. 11B). The overexpressing line, at the opposite of the knockout mutant, seemed to be disfavored in the regulation of water losses. Finally, *Attre1<sup>OE</sup>* line did not differ in its responses to MWD compared to Col-0 (Fig. 11). Indeed, this line had comparable transpiration rate to Col-0 and was also able to reduce it under MWD.

The results of this study suggest that trehalose content down regulates plant transpiration. A high level of trehalose inhibited transpiration while low trehalose content induced a larger transpiration rate in plants. Enhancement of water losses could explain the positive correlation between trehalose content and resistance to moderate water stress.



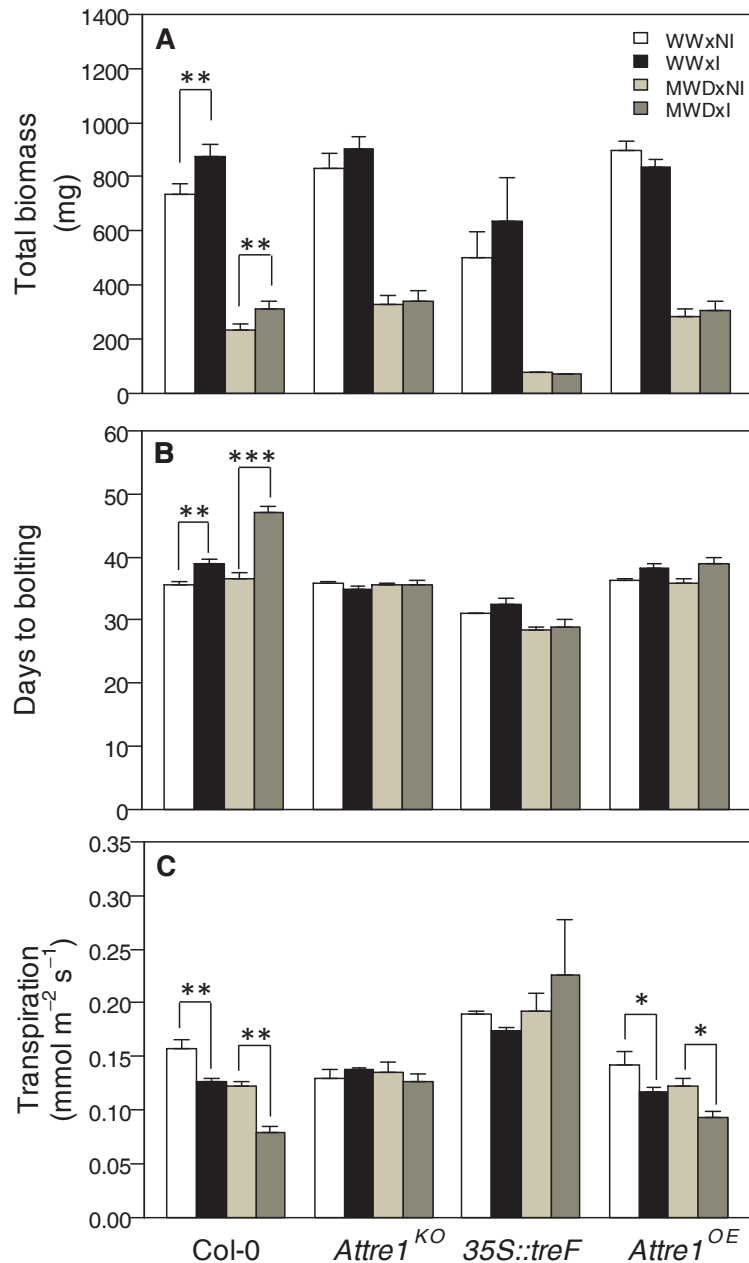
**Fig.12. Survival percentage of *TRE1*-modified mutants under severe water stress..** Asterisk indicate significant differences following Kruskal-Wallis test (\*\*  $P < 0.01$ ). Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>. The severe water deficit (SWD) was applied by progressive decrease of  $RWC_{soil}$  to 6%  $g\ H_2O\ g^{-1}$  dry soil by stopping of watering, then irrigation was restarted to reach the control soil water content (WW; 35%  $g\ H_2O\ g^{-1}$  dry) by daily adding a constant volume of a modified one-tenth-strength Hoagland solution (Hoagland & Arnon, 1950)

### II.2.3. *Attre1<sup>KO</sup>* lines are more sensible to severe drought stress than the other trehalase-modified lines

SWD induced a decrease in plant survival by 40% in Col-0 (Fig. 12). Very surprisingly, transgenic plants *Attre1<sup>KO</sup>* lines presented a great decrease in plant survival (Fig. 12). This result was consistent with a recent study showing that *Attre1<sup>KO</sup>* is more sensitive to severe drought stress (Van Houtte *et al.* (2013b). These authors showed that *Attre1<sup>KO</sup>* mutants lose water faster than wild-type plants, due to a lower sensitivity toward ABA-dependent stomatal closure. These results are also consistent with the observations in other modified lines in trehalose metabolism. For instance, the *35S::TPS1* lines (more trehalose, more T6P) exhibited ABA-insensitive phenotype (Avonce *et al.*, 2004) and non-embryo-lethal *tps1* mutants, which display 2-fold decrease in T6P content, are hypersensitive to ABA at the level of germination and stomata aperture (Gomez *et al.*, 2006).

In our study, over-expressing lines did not present different survival rates compared to Col-0 (Fig. 12). On the contrary, Van Houtte *et al.* (2013b) found that the overexpressing *AtTRE1* lines display a better recovery after drought stress than wild plants. The over-expressing plants have a better water-retaining capacity, through more a sensitivity to ABA. However, the trehalose and T6P content under severe water stress were not measured and it is possible that their contents were greatly affected under SWD. Further analyses of trehalose and T6P content under SWD are therefore necessary to drawing conclusions on the effect of trehalose on plant resistance to severe drought.

To conclude, many studies showed that it is possible to induce drought stress resistance in plants by manipulating genes involved in trehalose metabolism. However, the precise role of trehalose, and especially trehalase activity, in drought resistance remain unclear. The mechanisms allowing plants to cope with drought stress are numerous and are related to different strategies: escape, dehydration avoidance or tolerance to dehydration (see *Chapitre 1*). We showed that trehalose metabolism could have positive effects on mechanisms implicated in dehydration avoidance (i.e. under moderate water deficit) but opposite effects on mechanisms implicated in the tolerance to dehydration and plant survival (i.e. severe water stress leading to dehydration of tissues and plant mortality). Indeed, our experimentations suggest that accumulation of trehalose is a good method to avoid dehydration but a poor strategy to tolerate dehydration. In this hypothesis, the trehalose-induced resistance of plants would depend on the drought stress scenarios and on the genetic construction used.



**Fig.13. STM196 implication in plant responses to moderate water deficit.** (A) Total biomass, (B) days to reach bolting stage and (C) transpiration rate of plants harvested at bolting stage, inoculated (I) or not (NI) by STM196 under well watered condition (WW) and moderate water deficit (MWD). Asterisk indicate significant differences following Kruskal-Wallis test (\*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ ) between non-inoculated and inoculated plants: Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>. Transpirational water loss was determined by successive weighting of the pots over 3 days and nights (every 3 h approximately).

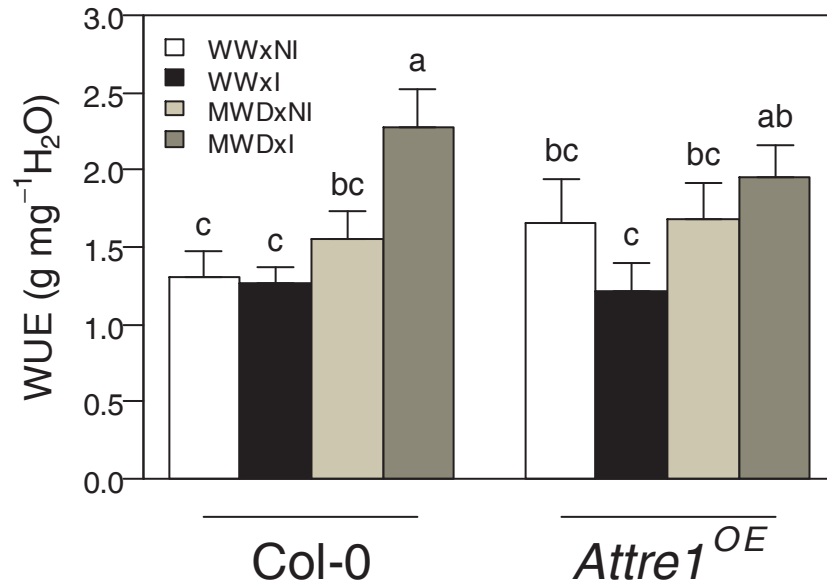
II.3. Unmodified *TRE1* expression is required for *Phyllobacterium brassicacearum* STM196 strain action on plant responses excepted under severe stress.

II.3.1. STM196 did not improve plant growth under WW and MWD conditions in transgenic lines.

*The developmental and physiological changes induced by STM196 in Col-0 were no longer observed in transgenic lines.*

We showed that STM196 promoted the biomass production of Arabidopsis Col-0 under well watered condition in soil (Fig. 13A;  $P < 0.01$ ). Under MWD, STM196 induced a better tolerance of wild type plants illustrated by the large increase in plant biomass (Fig. 13A;  $P < 0.01$ ). We showed that the improvement of plant tolerance to MWD is related to developmental changes such as reduction of initial growth rate and a delay in reproductive phase (see *Chapitre 3*). Indeed, STM196 induced a significant delay in flowering of Col-0 in both WW and MWD (Fig. 13B). Moreover, inoculated wild plants displayed a reduced transpiration rate regardless of soil condition (Fig. 13C;  $P < 0.01$  and  $P < 0.001$ , respectively). Coordinated changes in development and physiology of plants resulted in a higher water-use efficiency of inoculated plants (Fig. 14) and thus, in a better tolerance to water stress.

Surprisingly, the effects of STM196 on plant growth and physiology were not observed in the trehalase-modified mutants (Fig. 13). Under both WW and MWD conditions, the three lines did not present any modification on plant biomass when inoculated and thus, STM196 did not improved their tolerance to MWD (Fig. 13A). Moreover, mutants were not delayed in their flowering by inoculation (Fig. 13B and S3). At the physiological level, *Attre1<sup>KO</sup>* and *35S::treF* lines had similar transpiration rate with or without inoculation by STM196. On the contrary, *Attre1<sup>OE</sup>* lines presented the same responses than Col-0, i.e. a decrease in transpiration rate induced by STM196 (Fig. 13C). However, despite the changes in transpiration of *Attre1<sup>OE</sup>* lines by inoculation, this mutant did not display a better water use efficiency under MWD (WUE; Fig. 14).



**Fig.14. STM196 implication in plant responses to moderate water deficit.** Water use efficiency (WUE) of plants harvested at bolting stage, inoculated (I) or not (NI) by STM196 under well watered condition (WW) and moderate water deficit (MWD). Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ) between plants: Col-0, wild-type and the over-expressing lines *Attre1*<sup>OE</sup>. WUE was the amount of dry matter synthesized per unit of water lost, and was calculated as the ratio of absolute growth rate during the period of transpiration measurement to transpiration rate.

*STM196 inoculation did not induce strong modifications in trehalose metabolism in plants*

As unexpected, STM196 did not induced an increase in trehalose content, and tended to increase T6P content of Col-0 leaves without changes in T6P:trehalose ratio in plants harvested at bolting stage (Fig. 15A). Previous studies performed *in vitro* showed that STM196 increases trehalose and T6P leaf content in early developmental stages (i.e. 7 days after inoculation) through positive regulation of some *TPS* genes (Delteil *et al.*, in prep.). It is possible that the STM196 action on trehalose metabolism was different during the life cycle of plants, and that modifications that occurred early in plant development had repercussions on plant growth.

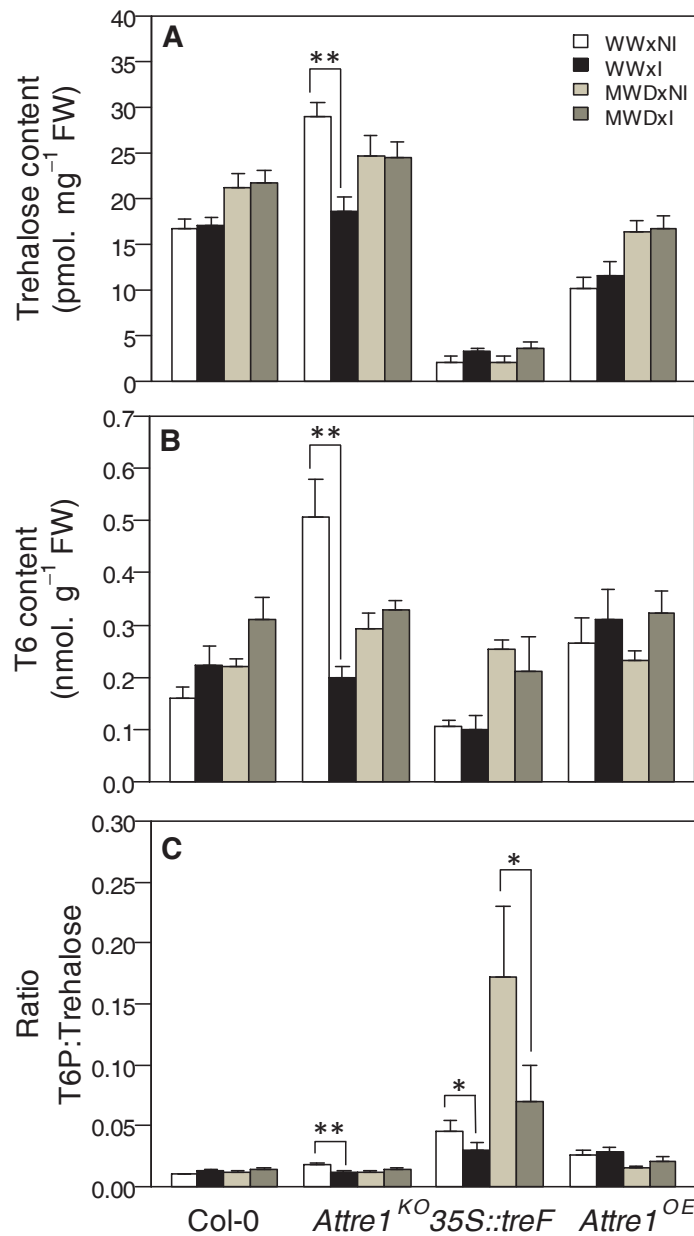
Interestingly, in transgenic lines, only *Attre1<sup>KO</sup>* lines presented modifications in trehalose and T6P content during STM196-inoculation under WW condition. Indeed, a decrease in trehalose and T6P content was observed in *Attre1<sup>KO</sup>* lines under WW condition, leading to a reduction of T6P:trehalose ratio (Fig. 15). STM196 seemed to alleviate the high trehalose and T6P contents in *Attre1<sup>KO</sup>*. We can hypothesize that STM196 would induce a glycosidase which degrades the trehalose content in plants when it gets over a threshold. Under MDW, STM196-inoculation did not affect a lot trehalose metabolism: the only difference concern the T6P:trehalose ratio in *35S::treF* which is decreased in plants inoculated by STM196 (Fig 15C).

STM196-inoculation does not induce PGPR effects on transgenic trehalase lines. This result cannot be explained by trehalose metabolism changes at bolting. However, we cannot exclude that unmodified trehalase expression or related modifications in plants were necessary for STM196 action on plants, and to improve plant tolerance to MWD.

*II.3.2. STM196 improved plant survival under SWD in all transgenic trehalase lines*

Under severe water stress, STM196 induced a significant increase in plant survival in Col-0 (*Chapitre 3*) but also in the three modified-trehalase mutants (Fig.16). This suggests that STM196 could have different actions on plant responses depending on the water deficit severity. However, it could be interesting to measure trehalose and T6P content in plants under different growing conditions in order to correlate the trehalose/T6P content in plants and efficiency of STM196.





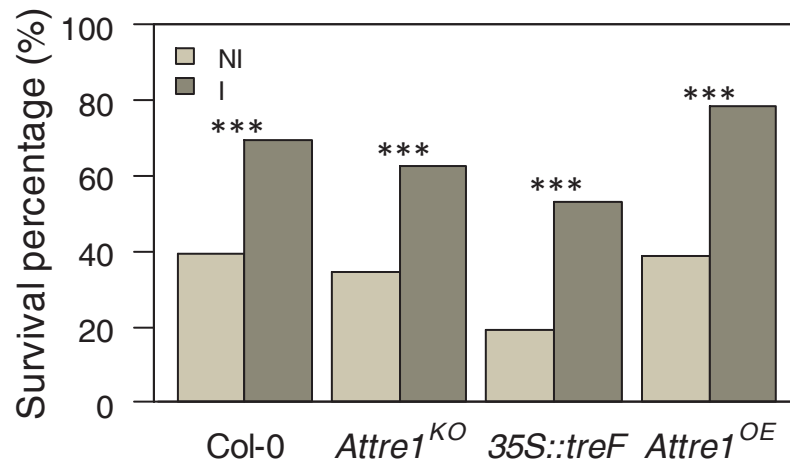
**Fig.15. STM196 implication in trehalose metabolism under moderate water deficit (A) Trehalose, (B) T6P content and (C) ratio T6P: trehalose in leaves of plants harvested at bolting stage under well watered condition (WW) and moderate water deficit (MWD), inoculated (I) or not (NI) by ST196. Asterisk indicate significant differences following Kruskal-Wallis test (\* $P < 0.05$  and \*\*  $P < 0.01$ ) between non-inoculated and inoculated plants: Col-0, wild-type, *Attre1<sup>KO</sup>* the knockout mutant and the over-expressing lines *35S::treF* and *Attre1<sup>OE</sup>*. Trehalose and T6P measurements were executed as Lunn *et al.* (2006).**

II.3.3. How to explain why STM196 was not able to promote growth in transgenic trehalase lines grown in WW and MWD conditions?

One of the most interesting results was the insensitivity of the modified-trehalase lines to the beneficial effects of STM196. Some hypotheses can be proposed in regards to this result.

**Hypothesis 1: Unmodified-trehalase expression is required for PGPR recognition by plants.** Trehalase has been reported to be important for interaction between plants and pathogen fungi (Brodmann *et al.*, 2002) or rhizobia (Muller *et al.*, 2001). It has been shown that inoculation by STM196 induced *TRE1* expression in roots but not in the shoot of *Arabidopsis* cultivated *in vitro* (Delteil *et al.*, in prep). In this way, trehalase could be a crucial enzyme that participates to the recognition between plants and STM196. Quantification of the effect of STM196 on *TRE1* expression in the different trehalase lines would bring some indication regarding this question.

**Hypothesis 2: STM196 does not survive in the rhizosphere of transgenic trehalase lines.** The lack of effects by STM196 on development and growth of plants may suggest that STM196 did not survive in the rhizosphere of modified-trehalase mutants. Since carbon metabolism in mutants is largely affected, we can postulate that transgenic lines produced modified root exudates that could repel the bacteria. Some studies reported that plants produce a variety of root exudates depending of genotypes that lead to a selectivity of the bacteria found in the rhizosphere (Micallef *et al.*, 2009; Chamam *et al.*, 2013). For this purpose, we assessed the survival of STM196 during soil drying and after rewatering, and we showed that growth of bacteria in the rhizosphere was not affected in presence of transgenic lines compared to Col-0 (data not shown). However, we don't know if the nature of root exudates of the transgenic lines allows or not the recognition between the plants and STM196.



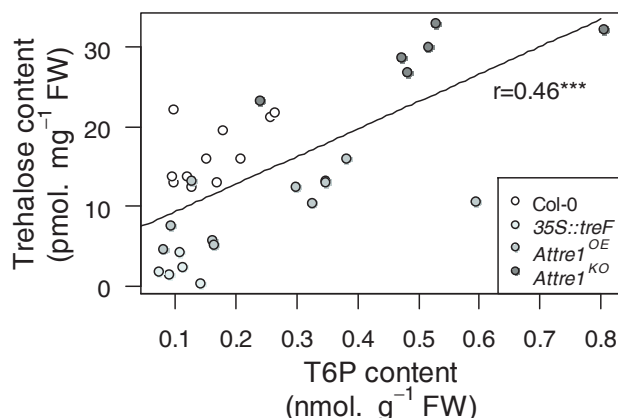
**Fig.16. STM196 implication on plant survival under severe water stress.** Survival percentage of plants inoculated (I) or not (NI) by STM196 under severe water. Asterisk indicate significant differences following Kruskal-Wallis test (\*\*\*)  $P < 0.001$ ) between non-inoculated and inoculated plants: Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>.

### *III. Conclusion*

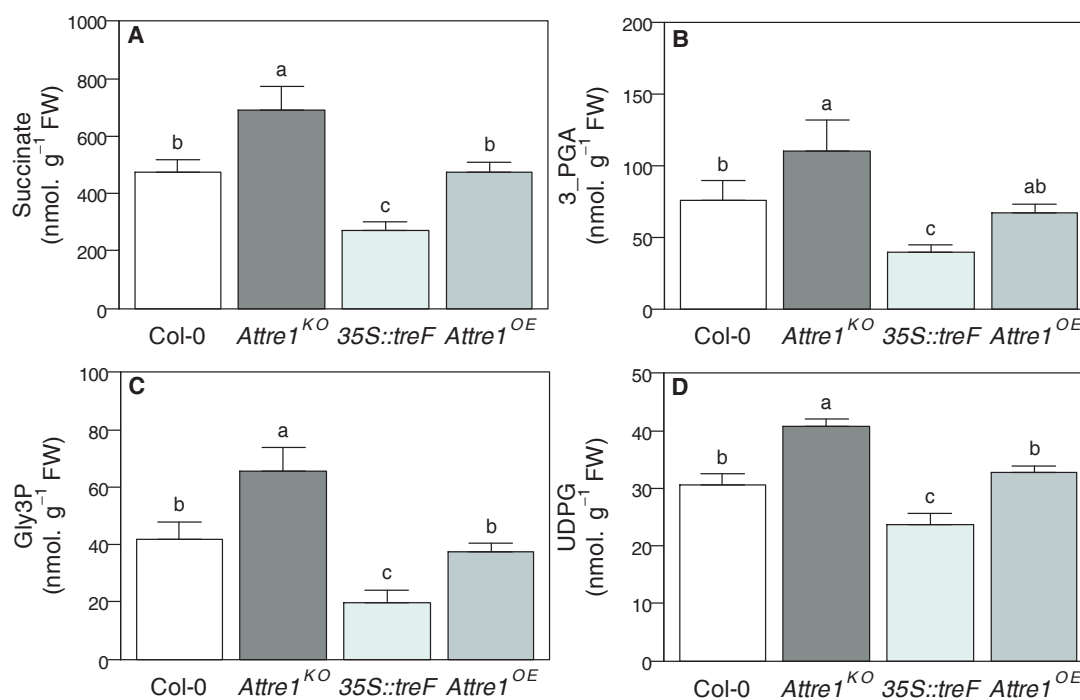
To understand the implication of trehalose metabolism in plant drought resistance and in the interaction between plants and PGPR, we used three trehalase-modified mutants. These lines displayed impaired trehalose metabolism with many pleiotropic effects. They were contrasted in terms of trehalose and T6P content and they also presented modifications in flowering time, growth and carbohydrate metabolism. Under water stress, the modified-trehalase expression had opposite effects depending on stress severity and thus, participated differentially in plant strategies to cope with water stress. Trehalase was implicated in complex regulations in plants, which complicated the understanding of action of inoculation by STM196. Under moderate water deficit, unmodified trehalase expression was essential for plant responses to inoculation by STM196, but seemed to be less implicated under severe water stress. Interestingly, STM196 seemed to have the same targets as the T6P/trehalose signaling (e.g., carbohydrate metabolism, delayed bolting time and plant growth promotion). The similarities of metabolic and physiological targets between STM196 and trehalose metabolism could suggest that STM196 modifies the metabolism and the physiology of plants via trehalose metabolism.

Improving production of plant biomass or plant survival under drought is difficult to obtain by genetic engineering. In our study, we showed that disrupting trehalase expression could be interesting in order to improve plant growth under moderate water deficit but was inappropriate under severe stress. However, inoculation by STM196 seemed to be more efficient to induce plant survival to drought than genetic manipulation of plants and could be a good strategy to promote plant growth under abiotic stresses.

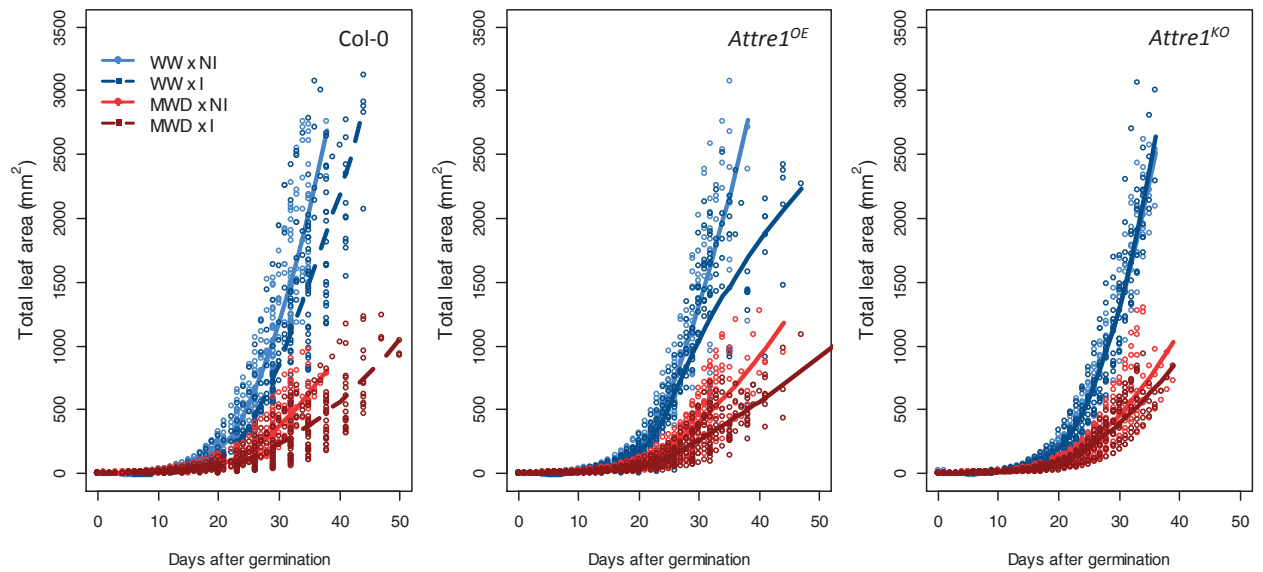
## Supporting information



**Fig.S1. Trehalose and T6P correlation.** Trehalose and T6P measurements were executed as Lunn *et al.* (2006) in plants harvested at the bolting stage in the middle of the day. Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>



**Fig.S2. Metabolite measurements in *TRE1*-modified mutants.** (A) Succinate, (B) 3-Phosphoglyceric Acid (3-PGA), (C) Glycerol-3-Phosphate (Gly3P) and (D) Uridine-diphosphate-glucose (UDPG) contents in leaves of plants harvested at the bolting stage in the middle of the day. Metabolites measurements were executed as Lunn *et al.* (2006). Letters indicate significant differences following Kruskal-Wallis test ( $P < 0.05$ ). Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing lines *35S::treF* and *Attre1*<sup>OE</sup>.



**Fig.S3. The growth of *TRE1*-modified mutants.** Total leaf area during days after germination of Col-0, wild-type, *Attre1*<sup>KO</sup> the knockout mutant and the over-expressing line *Attre1*<sup>OE</sup> inoculated (I) or not (NI) by STM196 under well watered condition (WW) and moderate water deficit (MWD). Projected area of the rosette were determined every 3 d from semiautomated analysis (ImageJ 1.43C; Rasband, Bethesda, MD, USA) of zenithal images of the plants.

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## ***Conclusion***

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## Conclusion

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En conditions naturelles, les plantes se développent en présence des microorganismes du sol qui peuvent coloniser l'intérieur et l'extérieur des racines. En fournissant de nouvelles voies nutritionnelles, influençant les voies biochimiques et améliorant les défenses des plantes contre des attaques environnementales, les rhizobactéries peuvent fondamentalement modifier le phénotype des plantes. L'impact de certaines de ces bactéries, en particulier les bactéries dites PGPR, sur la croissance des plantes et leur résistance à des stress abiotiques (e.g., stress hydrique, salin ou métallique) a été largement étudié (pour revues, voir Lugtenberg & Kamilova, 2009; Yang *et al.*, 2009; Friesen *et al.*, 2011). Cependant, malgré les connaissances acquises, peu d'études se sont intéressées à l'implication des PGPR sur le développement et la physiologie des plantes lors d'un stress hydrique. De nombreuses études ont pu mettre en évidence l'implication des PGPR dans la signalisation hormonale (Yang *et al.*, 2009) conférant aux plantes des ajustements plastiques favorables à leur résistance à des stress hydriques. De plus, les études sur les interactions plante-rhizobactérie généralement réalisées *in vitro* s'intéressent aux effets de l'inoculation lors des stades précoces du développement des plantes, plus particulièrement sur la croissance racinaire (pour revue, voir Vacheron *et al.*, 2013). Quelques études réalisées en sol, ont présenté l'impact de l'inoculation sur la croissance des plantes à un temps donné après germination ou inoculation (e.g., Creus *et al.*, 2004; Mayak *et al.*, 2004; Jaleel *et al.*, 2007; Zahir *et al.*, 2008)

Dans ce contexte, l'objectif de la thèse était d'approfondir les connaissances sur les interactions PGPR-plantes avec une approche intégrative sur la croissance et la physiologie des plantes en conditions stressantes et non stressantes. Pour cela, nous avons utilisé le couple *Arabidopsis thaliana*-*Phyllobacterium brassicacearum* STM196, qui présente l'avantage d'avoir été préalablement étudié *in vitro*. La souche STM196 induit des mécanismes communs aux PGPR chez *A.thaliana*, notamment dans la nutrition azoté, la croissance du système racinaire, la signalisation auxinique et l'éthylène (Mantelin *et al.*, 2006; Contesto *et al.*, 2008; Contesto *et al.*, 2010; Galland *et al.*, 2012; Kechid *et al.*, 2013) et permet ainsi un

transfert des connaissances acquises sur le rôle des PGPR. Dans cette étude, nous avons tiré parti de la plateforme de phénotypage PHENOPSIS (Granier *et al.*, 2006), qui permet une analyse fine et détaillée de la croissance et du développement d'Arabidopsis dans des conditions de culture quantifiées et maîtrisées, pour développer une nouvelle approche d'analyse à haut débit (nombreux individus) de l'effet des PGPR sur la plasticité phénotypique des plantes. Nous avons constaté qu'il est nécessaire d'analyser de façon dynamique l'effet des PGPR sur la croissance et le développement des plantes pour ne pas faire des conclusions biaisées (Lièvre *et al.*, 2013- *Chapitre 2*). En effet, dans notre étude, 41 jours après germination des plantes, l'effet de STM196 semblait avoir un effet négatif sur la croissance des plantes avec une réduction de la biomasse des plantes inoculées par rapport aux plantes non-inoculées. Cependant, à floraison les plantes inoculées ont présenté une augmentation significative de la biomasse des plantes, ce qui a révélé l'effet PGPR de la souche STM196 en condition en sol. L'analyse du développement d'Arabidopsis au cours du temps nous a permis de montrer que STM196 induit un retard de floraison et un ralentissement de la croissance des plantes (Bresson *et al.*, 2013; Lièvre *et al.*, 2013- *Chapitres 2 et 3*). Ce résultat a été pour la première fois mis en évidence dans les mécanismes relatifs aux interactions plante-PGPR, et ouvre de nouvelles perspectives d'analyse de l'effet promoteur de la croissance par les PGPR.

Nous nous sommes plus particulièrement intéressés à l'implication de STM196 dans les réponses des plantes au stress hydrique édaphique. Afin d'étudier l'effet de STM196 sur la diversité des mécanismes relatifs aux différentes stratégies de résistance des plantes, deux scénarios contrastés de stress hydrique ont été utilisés. La sévérité des stress a été définie en fonction de l'importance de leur effet sur l'état physiologique des plantes. Dans cette étude, le stress hydrique dit modéré a induit une très faible diminution de la teneur en eau des feuilles bien que la croissance ait été réduite de moitié, et le stress sévère a provoqué une forte déshydratation des tissus allant jusqu'à la mort des plantes. L'implication de STM196 dans les stratégies d'adaptation des plantes au stress hydrique a ainsi pu être étudiée.

L'inoculation par STM196 a montré que les rhizobactéries peuvent interférer dans les mécanismes d'échappement à la sécheresse des plantes. L'échappement à la sécheresse est défini par l'ajustement du développement des plantes au cours du temps, notamment par des modifications du temps de floraison, afin d'avoir un cycle de vie complet en évitant ou en diminuant l'impact du stress (Verslues & Juenger, 2011; Assmann, 2013). Chez *A. thaliana*, il existe une variabilité de réponses en condition de stress hydrique : par exemple Col-0 présente

une floraison plus précoce, alors qu'An-1 possède une floraison retardée (Vile *et al.*, 2012-Annexe 2). Ici, l'inoculation par STM196 a induit un retard de floraison chez *A. thaliana* en condition contrôle et avec un effet plus prononcé en condition de stress hydrique modéré. Ce retard de floraison, relié à un ralentissement de la croissance et à un décalage de la transition florale, a induit une meilleure résistance d'*A. thaliana* lors d'un stress hydrique modéré (Bresson *et al.*, 2013- Chapitre 3). Cependant, le décalage de la floraison des plantes dépend aussi de l'occurrence, de la durée et de la sévérité du stress (McMaster *et al.*, 2009). En présence d'un stress sévère ponctuel, un retard de la floraison de 20 jours a été observé chez Col-0 (Chapitre 4). L'inoculation par STM196 en condition de stress sévère n'a pas induit de modification du temps de floraison, malgré une amélioration de la tolérance des plantes à la déshydratation (Chapitre 4). STM196 semble donc induire des modifications de la phénologie selon le type de stress appliqué. A notre connaissance, l'implication des PGPR dans les mécanismes d'échappement n'est pas à ce jour connue. Quelques études réalisées en conditions non stressantes ont montré l'effet de rhizobactéries sur le temps de floraison chez *Arabidopsis*, et plus particulièrement une précocité de la floraison (Schwachtje *et al.*, 2011; Poupin *et al.*, 2013). La floraison est un évènement clé dans le développement des plantes qui détermine la production de biomasse des plantes (Jung & Muller, 2009). Le nombre de jours pour atteindre la floraison est un trait important dans la sélection de nouvelles variétés plus performantes aux champs (Salehi *et al.*, 2005; Korves *et al.*, 2007; Jung & Muller, 2009; Demura & Ye, 2010). Notre étude offre donc des perspectives intéressantes d'une part pour la connaissance des mécanismes d'action des PGPR sur la floraison des plantes, et d'autre part sur l'utilisation des interactions plantes-PGPR pour l'amélioration du rendement des cultures lors de contraintes hydriques.

STM196 intervient également dans les mécanismes impliqués dans l'évitement à la déshydratation des plantes lors d'un stress modéré (Bresson *et al.*, 2013- Chapitre 3). Lors du stress hydrique modéré, les plantes ont conservé un statut hydrique tissulaire optimal et compatible avec un bon fonctionnement métabolique des plantes, en présence ou non de STM196 dans le sol. Cependant, les plantes inoculées ont présenté une meilleure résistance au déficit hydrique, illustrée par un gain de biomasse de 50%, et une meilleure efficacité de l'utilisation de l'eau. L'inoculation par STM196 induit une coordination des mécanismes allant tous dans le sens d'une optimisation du prélèvement de l'eau dans le sol et une réduction des pertes d'eau via le système foliaire. En effet, d'une part STM196 a permis une augmentation du système racinaire permettant une exploration plus importante du sol.



D'autres parts, STM196 a induit une réduction des pertes en eau par une diminution de la transpiration via une augmentation de l'ABA dans les feuilles, un ralentissement de la croissance permettant de réduire les surfaces transpirantes, et une meilleure gestion du "budget" en eau.

STM196 interfère également dans la tolérance à la déshydratation des plantes. Lors du stress hydrique sévère, le statut hydrique des plantes a été fortement affecté, et a été diminué à des seuils incompatibles avec la vie des plantes (*Chapitre 4*). Dans cette condition drastique, l'inoculation par STM196 a permis une forte augmentation de la survie des plantes. En effet, STM196 a induit une meilleure tolérance à la déshydratation via un retard dans la déshydratation des tissus végétaux. De plus, STM196 a également permis une meilleure survie à d'importants dommages du photosystème II.

Par la suite, nous nous sommes intéressés au tréhalose comme molécule signal dans les réponses occasionnées chez les plantes suite à l'inoculation par STM196 (*Chapitre 5*). Le rôle du trehalose et de son précurseur le trehalose-6-phosphate, a été largement étudié dans le développement, le métabolisme et la résistance des plantes à des stress abiotiques (Paul, 2007). De plus, l'implication du tréhalose dans les interactions plant-microorganismes a été mise en évidence, notamment dans les symbioses entre légumineuses et rhizobactéries (Fernandez *et al.*, 2010). Ici, l'approche fonctionnelle sur le comportement de mutants affectés dans le métabolisme du trehalose, a montré que la plupart des effets de STM196 conférant une meilleure tolérance des plantes à un stress hydrique modéré disparaissait chez ces mutants. Ce résultat souligne l'implication du trehalose dans les réponses Arabidopsis lors de l'inoculation par STM196. A l'inverse, nos travaux montrent que l'inoculation par STM196 permet une amélioration de la survie des mutants. L'implication du trehalose dans les réponses des plantes à l'inoculation semble dépendre de la sévérité du stress appliqué aux plantes.

STM196 induit donc des changements à différentes échelles d'organisation, qui permettent une amélioration de la résistance des plantes dans des environnements très contrastés. L'inoculation par STM196 représente ainsi une valeur ajoutée dans les stratégies de résistance intrinsèques aux plantes. Ce résultat présente un intérêt dans un contexte agronomique étant donné la difficulté d'améliorer la résistance des plantes à la sécheresse par les voies classiques de la sélection génomique. En effet, un trait peut être bénéfique pour la résistance des plantes dans une condition et néfaste pour un autre type de stress (Tardieu,

2012). Par exemple, optimiser la fermeture stomatique, induisant une réduction de la transpiration, est un avantage non négligeable dans les résistances des plantes à un stress hydrique modéré. Cependant, une fermeture stomatique prolongée induit une diminution de l'assimilation du carbone et un échauffement des tissus lors d'un stress hydrique sévère ou avec une durée prolongée (McDowell, 2011; Tardieu, 2012). L'inoculation par des PGPR semble être une alternative pour l'optimisation du rendement des cultures dans des situations fluctuantes présentes aux champs. Les PGPR induisent des modifications globales du fonctionnement, qui sont le fruit de milliers d'années de coévolution entre plante et bactéries, passant par des mécanismes variés et pouvant être applicables à différents stress.

L'utilisation des PGPR a été intégrée dans l'agriculture et est reconnue pour son action dans l'amélioration des cultures (pour revue, voir Lucy *et al.*, 2004; Figueiredo *et al.*, 2011). Des formulations à base de mélange de PGPR sont de nos jours commercialisées et trouvent des usages en agriculture et en horticulture (Reddy *et al.*, 2001). Des études sur l'utilisation de mélange de PGPR ont été menées en serre ou en champs (Figueiredo *et al.*, 2008). Par exemple, lors d'un stress hydrique, la co-inoculation chez le haricot (*Phaseolus vulgaris* L.) par un mélange entre *Rhizobium tropici* et deux autres souches de *P. polymyxa* permet une meilleure stimulation de la croissance des plantes que lorsque les plantes sont inoculées seulement avec *Rhizobium tropici* (Figueiredo *et al.*, 2008). La co-inoculation par différentes rhizobactéries semblent un enjeu important.

Le Centre technique interprofessionnel des oléagineux et du chanvre (CETIOM) a effectué des expérimentations sur la culture de colza avec STM196 en conditions naturelles. L'utilisation de STM196 en champs s'est révélée décevante (CETIOM, Rapport d'activités 2003). Cependant, nous avons montré que les effets induits chez les plantes par STM196 sont exacerbés en déficit hydrique. Il serait donc intéressant de réaliser de nouvelles études en conditions de sécheresse. De plus, les travaux récents sur les PGPR (Figueiredo *et al.*, 2008) suggèrent qu'il serait intéressant d'étudier l'effet de STM196 en présence d'autres bactéries sur la croissance des plantes afin d'optimiser son action en champs.

Dans ce but, nous avons réalisé une étude préliminaire avec des souches de rhizobactéries non connues pour leur effet sur la croissance et la survie des plantes d'*A. thaliana* (Annexe I). Cette étude nous a permis de montrer la diversité des effets bactériens sur les réponses d'*Arabidopsis* au stress hydrique. Toutes les bactéries ont révélé un effet promoteur de la croissance des plantes lors d'un stress hydrique modéré. Cependant, certaines

bactéries ont été bénéfiques à la survie des plantes et d'autres négatives en condition de stress hydrique sévère. Outre la diversité des bactéries, il serait intéressant d'évaluer la variabilité des réponses des plantes, en profitant notamment des formidables ressources génétiques disponibles chez *A. thaliana*. Par ailleurs, l'utilisation des outils d'analyse génétiques (e.g., analyses QTL, génétique d'association) pourrait permettre d'identifier les gènes et les voies métaboliques impliqués dans les réponses des plantes au stress hydrique en présence de PGPR.

L'étude exhaustive de l'effet des communautés bactériennes sur la croissance végétale dans des environnements extrêmement variables est clairement un objectif inaccessible. Néanmoins il est maintenant possible d'étudier et de comparer les communautés bactériennes présentes dans la rhizosphère des plantes par des approches de métagénomique. Les travaux de cette thèse ont permis d'identifier les mécanismes adaptatifs clefs mis en jeu lors d'interactions avec l'environnement biotique et abiotique. Elle est une première étape dans le but d'évaluer la diversité et l'importance des interactions plantes-bactéries, un réel challenge pour transformer la productivité agricole, ainsi que pour comprendre le fonctionnement des écosystèmes naturels.

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## ***Annexe 1***

***Impact de la diversité des  
microorganismes sur la croissance  
d'A. thaliana en situation  
de stress hydrique :***

***Analyse de 6 bactéries***

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# ***Impact de la diversité des microorganismes sur la croissance d'*A. thaliana* en situation de stress hydrique : Analyse de 6 bactéries***

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(Stage de Master 1 ; Université de Montpellier II ; 4 mois)

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Les déficits hydriques réduisent la productivité des végétaux. Dans un contexte de réchauffement climatique, il semble crucial de comprendre les mécanismes mis en place par les plantes face à de tels stress abiotiques. L'interaction de bactéries avec la rhizosphère de la plante peut s'avérer bénéfique pour le développement de cette dernière qui peut lui conférer une protection face à un stress abiotique ou l'attaque de pathogènes : on parle alors de bactéries PGPR (Plant Growth Promoting Rhizobacteria). Le genre *Bradyrhizobium*, dont l'interaction symbiotique avec des Légumineuses a été largement étudiée, partage des mécanismes communs avec les PGPR décrites dans la littérature, notamment la synthèse de phytohormones. Dans cette étude, l'impact de 6 souches bactériennes sur la croissance et le développement de la plante modèle *Arabidopsis thaliana* (écotype Col-0) a été testé en condition de stress hydrique ou non : 4 souches de *Bradyrhizobium* provenant de sols sénégalais et deux souches au potentiel PGPR déjà observé chez *Arabidopsis* : *Pseudomonas fluorescens* et *Phyllobacterium brassicacearum*. Après avoir analysé les caractéristiques intrinsèques des différentes souches bactériennes, l'effet de chaque souche a été testé dans un système *in vitro* et dans un système en terre. Les changements morphologiques observés sur les plantes témoignent d'une interaction significative avec les bactéries dans les deux systèmes de culture. Les souches testées influencent la structure du système racinaire d'*Arabidopsis*, notamment *P. brassicacearum* dont les effets observés ont déjà été décrits. D'autre part, *P. fluorescens* semble conférer à la plante une résistance au stress hydrique appliqué *in vitro*. Enfin, les *Bradyrhizobia* ont un potentiel de stimulation de production de biomasse qui semble être corrélé dans les deux systèmes. Ces résultats ouvrent des perspectives de génomique fonctionnelle pour comprendre plus finement les mécanismes mis en jeu dans l'interaction plante/bactérie.

## I. Introduction

### I.1. Effets du stress hydrique sur le développement des plantes

Le réchauffement climatique actuel a un impact conséquent sur la disponibilité en eau (IPCC 2007). Les écosystèmes subissent ainsi des carences hydriques notamment dans les pays du Sud. Le déficit hydrique représente un stress abiotique qui a un effet néfaste sur la croissance et le développement des plantes. Dans un tel contexte, il est important de comprendre les stratégies déployées par les plantes pour faire face à un stress qui freine à la fois leur croissance et leur productivité (Boyer, 1982 ; Ciais *et al.*, 2005). La sécheresse affecte de nombreux processus chez la plante. Au niveau macroscopique, la production et l'expansion des feuilles de la plante sont fortement diminuées en réponse à un déficit hydrique (Taiz et Zeiger, 2006) alors que la teneur en matière sèche tend à augmenter. L'utilisation du carbone et de l'azote, éléments essentiels à la plante, notamment pour la photosynthèse, est également perturbée par les déficits en eau (Zhang *et al.*, 2008). Le développement du système racinaire est généralement favorisé par rapport aux parties aériennes, ce qui se manifeste par une réduction du rapport parties aériennes/parties souterraines (Chaves *et al.*, 2002). L'adaptation des plantes à la sécheresse se présente sous trois formes. Le mécanisme d'échappement est observé dans le cas de plantes à cycle court qui achèvent leur cycle avant l'arrivée de la sécheresse. En cas de stress hydrique prolongé, leur potentiel biologique peut être conservé sous la forme d'un organe (graine, bulbe, rhizome...) qui garde la capacité de germer lors de conditions favorables. Certaines plantes augmentent leur résistance à la déshydratation grâce à leur capacité à améliorer l'absorption d'eau et à en minimiser les pertes. A court terme, la fermeture des stomates limite les pertes en eau par transpiration. Sur le plus long terme, le développement d'un système racinaire étendu favorise un prélèvement efficace de l'eau contenue dans le sol (Farooq *et al.*, 2009). En outre, la diminution de la densité des stomates et de l'épaisseur de la cuticule pourrait favoriser l'étanchéité des feuilles (Shepperd et Griffiths, 2006). Mis à part ces changements phénologiques, morphologiques et anatomiques, des adaptations métaboliques permettent à la plante de supporter le déficit hydrique de leurs tissus, les mécanismes impliqués permettent d'induire une plus forte tolérance à la déshydratation. Face à un faible potentiel hydrique du sol, l'accumulation de solutés (sucres, potassium, acides aminés...) dans la vacuole et le cytoplasme des cellules foliaires entraîne une augmentation du potentiel hydrique des feuilles qui participe au maintien d'un statut hydrique favorable à la croissance de la plante. Cette osmorégulation maintient la turgescence

des cellules et l'apport en eau nécessaire au métabolisme de la plante. D'autres molécules peuvent être produites par la plante pour protéger ses cellules et en particulier ses membranes lors de la déshydratation : des protéines de type LEA (Late Embryogenesis Abundant), de la proline, du tréhalose, diverses molécules anti-oxydantes, des lipides (pour revue, Moore *et al.*, 2009).

## I.2. Effet des micro-organismes sur la croissance des plantes et sur leur résistance aux stress

Substrat naturel des plantes, le sol héberge une grande diversité de micro-organismes: bactéries, archaebactéries et champignons, qui participent au fonctionnement des écosystèmes. Les cycles biogéochimiques du carbone, de l'azote et du phosphore sont fortement dépendants de l'activité des micro-organismes telluriques. Cette flore microscopique joue un rôle essentiel dans le recyclage de la matière organique en libérant des éléments minéraux assimilables par les plantes. La capacité de certaines bactéries à fixer l'azote atmosphérique en font des fertilisants naturels des sols agricoles qui ont un impact indirect sur le rendement des cultures. La flore bactérienne qui abonde au niveau de la rhizosphère des plantes augmente la surface d'échange entre les racines et le sol. Les PGPR (Plant Growth Promoting Rhizobacteria) font partie de cette diversité bactérienne. Selon Kloepper et Schroth (1978), elles sont définies comme des bactéries capables de coloniser la rhizosphère et de stimuler la croissance de la plante par de nombreux mécanismes. Certaines PGPR ont notamment la capacité d'influencer la croissance de la plante en synthétisant des hormones végétales ou en modifiant la balance hormonale. L'association de ces rhizobactéries au niveau des parties racinaires améliore entre autres la nutrition azotée et en phosphate de la plante (Yang *et al.*, 2009, Dimpka *et al.*, 2009). Certaines PGPR confèrent aux plantes avec lesquelles elles interagissent une résistance aux stress biotiques ou abiotiques ; on parle alors de « bioprotection ». Leur influence sur la résistance des plantes au stress hydrique a déjà été étudiée (Creus *et al.*, 2004). Les mécanismes par lesquels les bactéries peuvent influencer la résistance à la sécheresse des plantes sont là aussi nombreux (pour revue, Dimpka *et al.*, 2009). Parmi ceux-ci, on peut noter la diminution de la production d'éthylène de la plante via l'activité ACC déaminase de la bactérie, l'augmentation du système racinaire via la biosynthèse de composés auxiniques par la bactérie ou encore la production de tréhalose.

### I.3. La biosynthèse de composés auxiniques et de tréhalose : deux mécanismes permettant aux bactéries d'influencer la croissance végétale en condition de stress hydrique

Kloepper et Schroth, en 1986, ont estimé qu'environ 80% des bactéries présentes dans la rhizosphère des plantes étaient capables de produire des composés auxiniques et en particulier de l'AIA (acide Indole Acétique). Les bactéries disposent en effet de tout un arsenal enzymatique leur permettant de synthétiser de l'AIA à partir de dérivés de tryptophane tels que l'acide indole-3-pyruvique, l'indole 3 acétaldehyde, la tryptamine ou l'indole-3-acétonitrile. Plusieurs études vont dans le sens de l'importance de cette production d'auxine dans les effets PGPR. Par exemple, des souches mutantes d'*Azospirillum brasilense* dans *IPDC* (Indole-3-pyruvate décarboxylase), gène avec un rôle majeur dans la capacité de production d'auxine chez cette bactérie, perd presque intégralement ses effets PGPR sur blé (Dobbelaere *et al.*, 1999). Une corrélation entre la capacité de produire de l'auxine par des bactéries isolées de la rhizosphère du blé et leur capacité à induire une augmentation de matière sèche des parties aériennes et souterraines du blé a été décrite par Khalid *et al.*, en 2004. Certains auteurs ont montré que l'action de certaines PGPR pouvait être mimée par l'ajout d'auxine dans le milieu de culture (Dobbelaere *et al.*, 1999). Enfin des analyses de transcriptomique montrent que des gènes de réponse à l'auxine sont induits chez *Arabidopsis* après inoculation par *Pseudomonas fluorescens* (Wang *et al.*, 2005). Tous ces résultats suggèrent donc que la capacité de production de l'auxine par les bactéries a un rôle majeur dans la modification de la croissance des plantes. Le rôle des faibles concentrations d'auxine sur la stimulation du développement du système racinaire, notamment sur l'élongation cellulaire et sur l'initiation de racines latérales, est souvent évoqué pour expliquer les effets de l'auxine relarguée par les bactéries sur la croissance des plantes, en conditions de stress hydrique ou non (Dimpka *et al.*, 2009).

Le tréhalose est un disaccharide non réducteur composé de deux glucoses liés par une liaison  $\alpha 1 \rightarrow 1$ . Chez les bactéries, les champignons, les insectes (dans l'hémolymphe) ou encore les plantes inférieures, le tréhalose est habituellement synthétisé en cas de stress abiotiques (Wingler, 2002). L'induction d'une surproduction de tréhalose dans les plantes par transformation génétique induit une résistance à la sécheresse; ceci a été clairement montré dans le cas du tabac (Romero *et al.*, 1997), du riz (Garg *et al.*, 2002; Jang *et al.*, 2003) ou d'*Arabidopsis thaliana* (Miranda *et al.*, 2007; Karim *et al.*, 2007) notamment. Chez les plantes supérieures, le tréhalose est en trop faible quantité ( $10 \mu\text{g g}^{-1}$  de matière fraîche) pour

pouvoir agir comme osmoprotectant, comme cela est le cas pour les plantes reviviscentes. Son rôle correspondrait plutôt à celui d'une molécule signal (Wingler, 2002) efficace lors de stress abiotiques. D'autres études suggèrent que les bactéries produisant une quantité importante de tréhalose confèrent aux plantes une résistance à des stress hydriques. L'inoculation par une bactérie de type *Rhizobium* surexprimant la tréhalose-6-phosphate synthase (TPS) permet au haricot de mieux supporter le stress hydrique (Suárez *et al.*, 2008). Des plants de maïs inoculés avec *Azospirillum brasiliense* surproduisant le tréhalose résistent mieux au manque d'eau (85% de survivants) et ont une biomasse plus importante de 73% que les plants inoculés avec la bactérie sauvage (55% de survivants) (Rodríguez-Salazar *et al.*, 2009).

#### I.4. Quelles bactéries sont susceptibles de modifier la croissance des plantes en conditions de stress hydrique ?

La diversité des bactéries vivant dans le sol est immense : vouloir étudier l'impact de la diversité bactérienne sur la croissance en condition de stress hydrique est donc un travail de longue haleine. Dans cette étude pionnière, il a fallu présélectionner un certain nombre de bactéries. Les critères principaux de choix ont été i) la capacité des bactéries à vivre en association avec des plantes et ii) le niveau de caractérisation des bactéries par différentes équipes du LSTM. Six souches bactériennes ont été choisies : 4 souches de *Bradyrhizobium*, *Phyllobacterium brassicacearum* (STM196) et *Pseudomonas fluorescens* (STM324). Les bactéries du genre *Bradyrhizobium* sont de bons candidats. Elles sont reconnues comme rhizobactéries et ont un rôle important pour l'agriculture de par leur capacité à fixer l'azote atmosphérique au cours de leur symbiose avec des plantes Légumineuses. Les quatre souches de *Bradyrhizobium* sélectionnées ont été isolées à partir de nodules racinaires chez le niébé (*Vigna unguiculata*), une légumineuse semblable au haricot cultivée sur les sols arides sénégalais. Les analyses par PCR-RFLP des IGS (intergenic spacer region) des ADNr 16S-23S ont permis de distinguer différents profils génétiques (Krasova-Wade *et al.*, 2003). Deux types d'IGS sont représentés parmi les 4 souches de *Bradyrhizobium* sénégalaises dont nous disposons : le type I (ORS3258) et le type VI (ORS3409, 3410 et 3411).

*Phyllobacterium brassicacearum* (STM196) a été isolée par J.C Cleyet-Marel à partir des racines de colza (*Brassica napus*). Cette bactérie augmente *in vitro* la densité et la longueur des racines latérales et des poils racinaires du colza *via* un mécanisme dose-dépendant (Larcher *et al.*, 2003). Des effets PGPR identiques ont été observés *in vitro* chez *Arabidopsis thaliana* (Mantelin *et al.*, 2006). Cet effet PGPR est indépendant de la capacité

de la bactérie à modifier la production d'éthylène des plantes : en effet une inoculation par une souche Acds-, et donc incapable d'utiliser l'ACC des plantes, donne les mêmes effets sur l'architecture racinaire et la croissance qu'un STM196 sauvage (Contesto *et al.*, 2008). D'autre part, STM196 produit très peu d'auxine mais semble capable de modifier la perception de l'auxine dans la plante (Contesto *et al.*, 2010). Enfin, les plantes d'*Arabidopsis* inoculées par cette bactérie accumulent du tréhalose dans leurs parties aériennes (Tasselli, 2007).

*Pseudomonas fluorescens* (STM324) a été isolée dans la rhizosphère de la tomate (*Solanum lycopersicum*). Il a été démontré que l'inoculation de *Pseudomonas fluorescens* chez *Catharanthus roseus* confère à la plante une protection face à un stress hydrique (Jaleel *et al.*, 2007).

Au cours de ce stage, il a été question de sélectionner des bactéries conférant à la plante modèle *Arabidopsis thaliana* L. des comportements contrastés vis-à-vis de sa réponse à différents stress hydriques modérés ou sévères. Après avoir caractérisé les différentes souches bactériennes étudiées et établi un protocole expérimental pour l'étude de l'interaction plante/bactérie *in vitro* et en sol, nous avons cherché à savoir si des bactéries symbiotes de légumineuses peuvent avoir un effet PGPR une fois inoculées chez une plante éloignée phylogénétiquement.

## II. Matériel et Méthodes

### II.1. Mise en place d'un protocole de culture et caractérisation de la croissance des différentes souches bactériennes

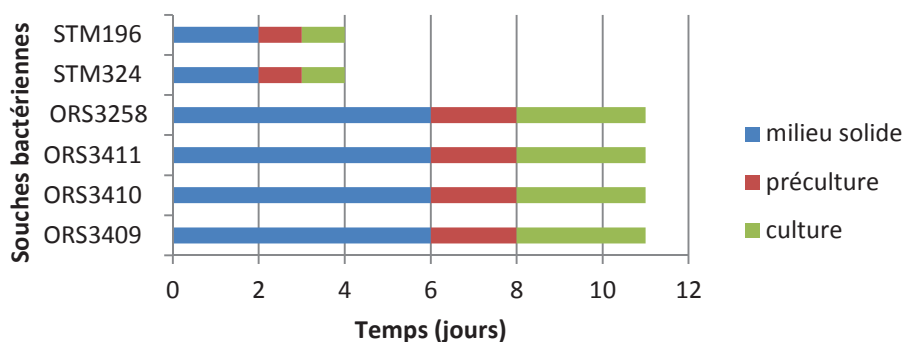
#### II.1.1. Souches bactériennes et conditions de culture

Les 6 souches bactériennes utilisées pour cette étude ont été fournies par le Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM, Montpellier, France, Table 1). Chaque souche a été cultivée individuellement sur une boîte de Pétri contenant le milieu gélosé adéquat. L'équivalent d'une anse de platine a été prélevée du stock glycérolé (conservation à -80 °C) puis étalé sur milieu gélosé semi-solide. *Phyllobacterium brassicacearum* (STM196) a été cultivé sur milieu E' (pH=6,8) composé de 2,9 mM K<sub>2</sub>HPO<sub>4</sub> ; 0,8 mM MgSO<sub>4</sub> ; 1,7 mM NaCl ; 7,9 mM KNO<sub>3</sub> ; 0,3 mM CaCl<sub>2</sub> ; 0,030 mM FeCl<sub>3</sub> ; 3 g.L<sup>-1</sup> d'extrait de levure ; 10 g.L<sup>-1</sup> de mannitol ; H<sub>2</sub>O milliQ qsp 1L ; 15 g.L<sup>-1</sup> d'agar. Les autres souches bactériennes ont été cultivées sur milieu Yeast Mannitol (YM) Agar (pH=6,8) contenant (par litre) : 1 g d'extrait

**Table 1. Souches bactériennes utilisées pour cette étude.**

Souche bactérienne <sup>1</sup>	Plante hôte d'origine	Origine géographique
<i>Bradyrhizobium</i> spp.		
ORS3258	<i>Vigna unguiculata</i>	Sénégal
ORS3409	<i>Vigna unguiculata</i>	Sénégal
ORS3410	<i>Vigna unguiculata</i>	Sénégal
ORS3411	<i>Vigna unguiculata</i>	Sénégal
<i>Phyllobacterium brassicacearum</i>		
STM196	<i>Brassica napus</i>	France
<i>Pseudomonas fluorescens</i>		
STM324	<i>Solanum lycopersicum</i>	-

<sup>1</sup> Désignations : ORS, collection de l'Institut de Recherche pour le Développement, Montpellier, France ; STM, collection du Laboratoire des Symbioses Tropicales et Méditerranéennes.

**Fig. 1. Temps nécessaire à chaque étape de culture des souches étudiées.****Table 2. Caractéristiques bactériennes déterminées à l'aide de dénombrements sur milieu semi-solide et des courbes de croissance en milieu liquide (cf Figures 2 et 3).**

Souche bactérienne <sup>1</sup>	Concentration bactérienne (ufc/mL) pour $DO_{595nm}=0,7$	$\lambda$ <sup>2</sup>	$\mu$ <sup>3</sup>	A <sup>4</sup>
<i>Bradyrhizobium</i> spp.				
ORS 3258	$5,7 \cdot 10^8$	31,18	0,05	4,27
ORS 3409	$5,1 \cdot 10^8$	38,66	0,07	5,57
ORS 3410	$9,6 \cdot 10^8$	33,11	0,07	5,01
ORS 3411	$4,3 \cdot 10^8$	28,63	0,05	4,03
<i>Phyllobacterium brassicacearum</i>				
STM196	$10^8$			
<i>Pseudomonas fluorescens</i>				
STM324	$2,6 \cdot 10^8$	5,11	0,11	3,46

<sup>1</sup> Désignations : ORS, collection de l'Institut de Recherche pour le Développement, Montpellier, France ; STM, collection du Laboratoire des Symbioses Tropicales et Méditerranéennes.

<sup>2</sup>  $\lambda$  : Durée de la phase de latence

<sup>3</sup>  $\mu$  : Taux de croissance maximal durant la phase exponentielle

<sup>4</sup> A : Valeur de la  $DO_{595nm}$  de la phase stationnaire



de levure ; 10 g de mannitol ; 0,5 g K<sub>2</sub>HPO<sub>4</sub> ; 0,409 g MgSO<sub>4</sub> (H<sub>2</sub>O)<sub>7</sub> ; 0,1 g NaCl; 10 g d'agar ; H<sub>2</sub>O milliQ qsp 1 L. Toutes les souches ont été cultivées en conditions de semi aérobie dans des boîtes de Pétri scellées au Parafilm et placées à l'obscurité à 30 °C (25 °C pour STM196). Après un temps donné (Fig. 1), les colonies formées sur le milieu semi-solide ont permis l'inoculation avec l'équivalent d'une anse de platine de 200mL de milieu liquide (sans agar) afin de réaliser une pré-culture en erlenmeyer de chaque souche. Les erlenmeyers de pré-culture ont été placés en condition aérobie sur table agitante (160rpm) à l'obscurité, à 30°C (25°C pour STM196). La croissance des bactéries en pré-culture a été suivie par densité optique (DO<sub>595nm</sub>). La culture de chaque bactérie s'est effectuée dans un volume plus conséquent destiné à l'inoculation de terre ou de milieu de culture *in vitro*. Ainsi, des bouteilles contenant 750mL de milieu liquide, inoculées avec 20mL de pré-culture bactérienne en phase exponentielle, ont été manipulées dans les mêmes conditions que pour les pré-cultures.

### II.1.2. Courbes de croissance bactérienne

La mesure régulière de la densité optique d'1 mL de milieu liquide inoculé a permis de suivre la croissance de chaque souche en milieu liquide (Fig. 2 et 3). Les courbes de croissance ont été ajustées avec le logiciel R en utilisant le package *grofit* (Fitting Biological Growth Curves with R) selon l'équation suivante :

$$y(t) = \frac{A}{1 + \exp\left(\frac{\mu}{\lambda}(\lambda - t) + 2\right)}$$

Différents paramètres ont pu être déterminés dont la durée de la phase de latence ( $\lambda$ ), le taux de croissance en phase exponentielle ( $\mu$ ) et la valeur de la DO<sub>595nm</sub> de la phase stationnaire (A) pour chacune des souches utilisées dans cette étude (Table 2). Chaque courbe de croissance a pu être modélisée à partir de 3 répétitions biologiques minimum.

### II.1.3. Dénombrement des souches bactériennes

Afin de connaître la concentration bactérienne (ufc/mL) présente dans 1 unité de DO, chacune des souches bactériennes a été dénombrée par la technique MPN (most probable number). Une série de dilutions d'une pré-culture de densité optique connue a été réalisée pour chacune des souches étudiées. Un volume de 100  $\mu$ L de pré-culture a été étalé sur boîte de Pétri contenant du milieu YM congo red semi-solide pour chaque dilution. Les boites ont été

placées en chambre de culture à 30°C et à l'obscurité. Les colonies formées ont ensuite été dénombrées après un temps donné selon le type bactérien. Les études antérieures réalisées sur STM196 ont révélé qu'une densité optique de 0,7 équivaut à  $10^8$  ufc/mL et ont permis de s'affranchir de son dénombrement. La concentration bactérienne des autres souches a été calculée pour une DO de 0,7 (Table 2).

## II.2. Caractérisation des bactéries

### II.2.1. Dosage des composés auxiniques chez les bactéries en présence ou non de Polyéthylène glycol

La production de composés auxiniques par les bactéries à partir de Tryptophane (Trp) a été évaluée par dosage colorimétrique selon la méthode de Salkowsky. 1 mL de culture bactérienne en phase exponentielle a été ajouté dans un falcon de 50 mL contenant 8mL de milieu YM avec ou sans Polyéthylène Glycol (PEG 8000 : Sigma, Saint-Louis, USA) à 13% et 1mL d'une solution de Trp à  $5\text{g.L}^{-1}$  filtrée sous hotte. Les falcons ont été placés sur table à agitation (160rpm) à 30°C. La densité optique des solutions a été évaluée à 590nm après 20h de culture. 2mL de chaque falcon ont été centrifugés à 7000rpm pendant 5 minutes afin de récupérer le surnageant qui a été placé à 4°C à l'obscurité. Une gamme étalon a été réalisée à partir d'une solution mère d'AIA à  $250\text{ mg.mL}^{-1}$ . 0,5mL de l'échantillon à doser a été mélangé à 0,5 mL de réactif de Salkowsky (0,12 mL  $\text{FeCl}_3$  ; 4,44 mL  $\text{H}_2\text{SO}_4$  et 5,56 mL  $\text{H}_2\text{O}$ ) avant d'en mesurer la densité optique à 530nm. Dix répétitions biologiques ont été effectuées pour chaque condition.

### II.2.2. Dosage du tréhalose chez les bactéries en présence ou non de Polyéthylène glycol

La teneur en tréhalose dans les tissus bactériens a été évaluée par dosage enzymatique. Pour cette expérience, les souches bactériennes ont été cultivées avec ou sans PEG à 13% dans les mêmes conditions que les pré-cultures (cf. Souches bactériennes et conditions de culture). Une fois la phase exponentielle atteinte, les pré-cultures ont été centrifugées 5 min à 7000 rpm. Le culot a été repris et rincé dans 1 mL d'eau osmosée et à nouveau centrifugé (10 min à 7 000 rpm). Le culot formé a été congelé à -80°C après élimination du surnageant. Le culot congelé a subi une série de chocs thermiques en alternant 10 min à 90°C et 2 min dans l'azote liquide. Le culot a été repris dans 100 $\mu$ L d'eau osmosée avant d'être centrifugé 5 min à 7000 rpm. Le tréhalose a été dosé grâce au kit Megazyme (K-TREH 01/09) selon le

protocole suivant : 10  $\mu\text{L}$  du surnageant obtenu a été mélangé à 1,9 mL d'eau distillée, 100  $\mu\text{L}$  d'imidazole, 50  $\mu\text{L}$  de  $\text{NADP}^+/\text{ATP}$  et 10  $\mu\text{L}$  d'hexokinase/glucose-6-phosphate déshydrogénase dans une cuve. L'absorbance du mélange a été mesurée à 340 nm. L'absorbance a été à nouveau mesurée 5 min après l'ajout de 10  $\mu\text{L}$  tréhalase. La concentration en tréhalose du surnageant dosé a été calculée en fonction des différents paramètres régissant ce dosage (Annexe 1). L'estimation de la masse sèche du culot, après 2 jours à l'étuve, a permis de normaliser la quantité de tréhalose présente dans le surnageant et de déterminer ainsi la teneur en tréhalose des tissus bactériens. Cinq répétitions biologiques ont été effectuées pour chaque condition.

### II.2.3. Test de survie des bactéries en milieu gélosé

A 15 jours après semis (jas), 3 carottes de milieu gélosé sur lequel ont été cultivées les plantules ont été prélevées pour chacune des conditions de culture (avec ou sans PEG). Après un broyage au pilon dans un tube Eppendorf de 1,5 mL en présence de 200  $\mu\text{L}$  de milieu YM, les solutions obtenues ont été soumises à agitation pendant 30 min. Un volume équivalent à 1 mg de la carotte prélevée a été étalé sur boîte de Pétri contenant un milieu de culture semi-solide adéquat pour chaque souche bactérienne. Après quelques jours de culture en semi-aérobie à l'obscurité, la présence de colonies bactériennes sur le milieu gélosé a été vérifiée.

### II.2.4. Influence du Polyéthylène glycol sur la croissance des bactéries en milieu liquide

Chaque souche bactérienne a été cultivée dans des erlenmeyers selon le protocole décrit plus haut (cf. Souches bactériennes et conditions de culture) en présence ou non de PEG à 13%. La densité optique d'1 mL de solution de culture a été mesurée pour les deux conditions au bout du même temps de culture et comparée l'une à l'autre afin de déterminer un éventuel effet du PEG (Fig. 4). 3 répétitions biologiques ont été effectuées pour chaque condition.

## II.3. Culture des plantes *in vitro*

### II.3.1. Conditions de culture des plantes *in vitro*

2 L de cultures bactériennes en phase exponentielle ont été centrifugées 5 min à 5000 rpm. Le culot a été repris dans 30 mL de milieu plante ( $\text{pH}=5,7$ ) composé de 0,5 mM  $\text{CaSO}_4 (\text{H}_2\text{O})_2$  ; 2 mM  $\text{KNO}_3$  ; 0,5 mM  $\text{MgCl}_2 (\text{H}_2\text{O})_6$  ; 1 mM  $\text{KH}_2\text{PO}_4$  ; 0,05 mM  $\text{Na}_2\text{FeEDTA}$  ; 2,5 mM MES ; Mix oligoéléments 1x (0,03 mM  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}(\text{H}_2\text{O})_4$ , 1 mM  $\text{CuCl}_2(\text{H}_2\text{O})_2$ , 1 mM

ZnCl<sub>2</sub>, 15 mM MnCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub>, 50 mM H<sub>3</sub>BO<sub>3</sub>) ; H<sub>2</sub>O milliQ qsp 1 L. Le milieu plante a été inoculé avec un volume de solution bactérienne suffisant pour obtenir une concentration bactérienne de 10<sup>8</sup> bactéries/mL. 40 mL de milieu plante inoculé ont été coulés dans chaque boîte de Petri. Afin de simuler un stress hydrique *in vitro*, certaines boîtes ont été recouverte de 40 mL de milieu plante additionné de PEG à 26% durant 24 h avant d'enlever le surplus n'ayant pas diffusé dans la gélose. La concentration finale de PEG dans la gélose se retrouve alors réduite de moitié (13%). Une rangée de 10 à 15 graines d'*Arabidopsis thaliana* L. (Heynh), écotype Columbia (Col-0), préalablement stérilisées en surface (eau de Javel 2%, goutte de Tween 20), a été semée à l'aide d'une micropipette. Dans le but de minimiser l'évaporation mais de permettre néanmoins les échanges gazeux, les boîtes ont été scellées avec du scotch (Micropore, Elkton, MD, USA). Les boîtes ont été placées en chambre froide (4 °C) à l'obscurité pendant une durée minimale de 24 h puis transférées en chambre de culture (21 °C, 16 h jour/ 8 h nuit, 20 000 lux) où les boîtes ont été placées à la verticale. Après germination, les boîtes ont été éclaircies pour ne laisser que 5 plantules par boîte.

### II.3.2. Caractérisations phénotypiques des plantes cultivées *in vitro*

Les mesures ont été effectuées 12 jours après semis (jas). 20 plantules par condition ont été prélevées à l'aide d'un scalpel par section au niveau du collet et pesées à l'aide d'une balance de précision (Adventurer Pro, Ohaus Corporation, Pine Brook, NJ, USA) afin de déterminer la masse fraîche foliaire (MF). Les boîtes de culture *in vitro* ont été scannées et analysées grâce au logiciel ImageJ et au module d'extension NeuronJ 1.01 (Erick Maijering, Suisse). La longueur de la racine primaire (racine I), le nombre et la longueur des racines secondaires (racines II) ont ainsi été déterminés pour 10 plantules par condition. La densité de racines secondaires présentes sur la racine primaire a été calculée en nombre de racines secondaires par cm de racine primaire. Les poils racinaires présents sur la racine I entre la coiffe et la racine II la plus proche de la coiffe ont été observés à l'aide d'une loupe binoculaire munie d'une caméra Olympus SZH10 (Olympus, Tokyo, Japon) et d'une caméra digitale analogique CDD JVC TK-C1381 (JVC, Japon). A partir des photographies obtenues, la longueur moyenne des poils racinaires a été déterminée (n = 40 poils) sur 20 plantules à l'aide du logiciel Cell P (Olympus-Soft Imaging System, Japon).

## II.4. Culture des plantes en terre

### II.4.1. Inoculation de la terre

Les cultures bactériennes en milieu liquide ont été arrêtées pendant la phase exponentielle de chaque souche afin de récupérer les cellules bactériennes par centrifugation (4 000 rpm pendant 10 min). Les culots ont été repris avec de l'eau osmosée avant d'estimer la quantité de cellules par densité optique à 595 nm. De la terre préalablement ionisée à 40 kGray aux rayons  $\gamma$  (Entreprise Ionisos, Dagneux, France) a été inoculée avec un volume déterminé pour chaque souche, de façon à obtenir une concentration bactérienne de  $5.10^7$  bactéries par gramme de sol.

### II.4.2. Conditions de culture des plantes en terre

Quatre graines d'*Arabidopsis thaliana* écotype Col-0 ont été semées dans des pots de 200 mL contenant un mélange (50/50) de terreau et de terre limono-argileuse stérilisé par ionisation aux rayons  $\gamma$ . La terre a préalablement été humidifiée et homogénéisée avant d'être répartie dans les pots. L'humidité relative du sol ( $HR_{sol}$ , exprimée en  $g\ H_2O.g^{-1}\ sol\ sec$ ) initiale a été calculée grâce à la perte d'eau d'échantillons de terre placés à l'étuve (60°C). Les graines ont été soumises à stratification en plaçant les pots en chambre froide pendant une durée minimale de 48 h. La germination a été initiée en disposant les pots dans une chambre de culture aux conditions contrôlées : 20 °C jour/17 °C nuit, 12 h30 de photopériode, 235  $\mu mol\ photons.m^{-2}.s^{-1}$ , VPD : 0,35 kPa.

Le stress hydrique a été appliqué à partir de l'apparition des deux premières feuilles (stade 1.02 ; Boyes *et al.*, 2001) en arrêtant l'irrigation du substrat de culture jusqu'à atteindre l'humidité relative souhaitée. L'humidité de la terre a été contrôlée par la pesée quotidienne de chaque pot par le robot de phénotypage haut-débit PHENOPSIS (Granier *et al.*, 2006) ou de façon manuelle. La condition de culture contrôle (35% soit 0,35  $g\ H_2O.g^{-1}\ sol\ sec$ ) a été respectée grâce à des réajustements réguliers de l'humidité du sol avec une solution nutritive de Hoagland diluée au 10<sup>ème</sup>. Les pots soumis à un stress hydrique modéré continu ont été privés de solution nutritive jusqu'à atteindre 20%  $HR_{sol}$ , valeur conservée jusqu'à la récolte. Les pots soumis à un stress hydrique drastique ponctuel ont été privés d'irrigation jusqu'à atteindre 7% d'humidité dans le sol, seuil à partir duquel le pot a été à nouveau irrigué pour atteindre l'humidité contrôle de 35%. 18 individus ont été cultivés en condition contrôle, 9 en condition de stress hydrique modéré et 9 pour le test de survie.

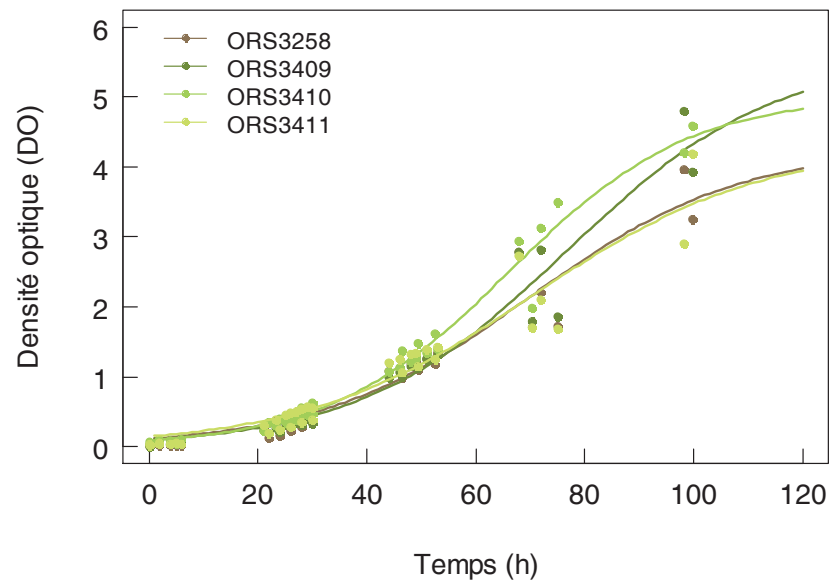


Fig. 2. Courbe de croissance des souches de *Bradyrhizobium*.

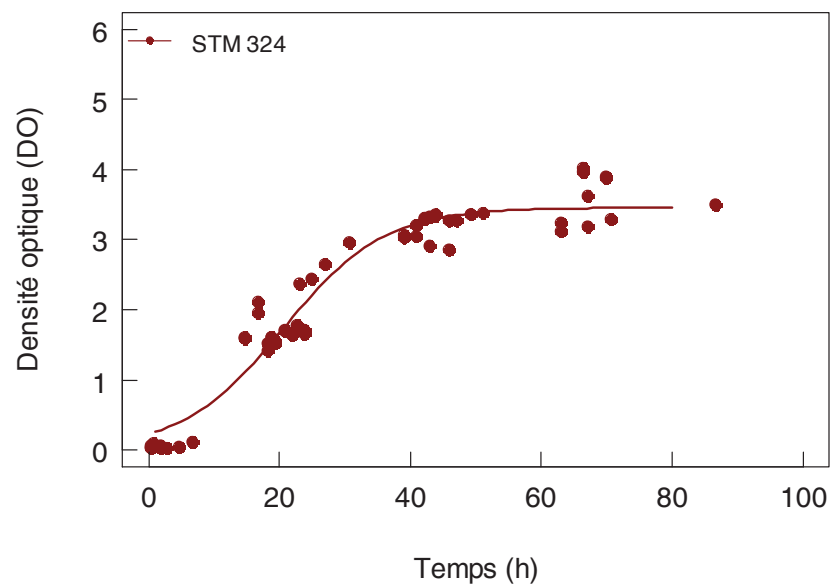


Fig. 3. Courbe de croissance de *Pseudomonas fluorescens* (STM324).

### II.4.3 Caractérisation phénotypique des plantes cultivées en terre

La récolte des plantes s'est effectuée au stade d'apparition des bourgeons floraux (stade 5.01, Boyes *et al.*, 2001). La matière fraîche foliaire a été évaluée juste après la récolte à l'aide d'une balance de précision. Les racines ont été extraites du substrat puis rincées dans de l'eau osmosée. Après 5 jours à l'étuve à 60°C, la masse sèche des racines a été déterminée à l'aide d'une balance de précision.

## II.5. Analyses statistiques

Les analyses statistiques ont été réalisées à l'aide du logiciel R. Des tests non paramétriques de comparaisons multiples de moyennes (Kruskal-Wallis) ont été réalisés sur l'ensemble des variables étudiées afin de comparer les souches bactériennes, leurs effets sur la croissance et la morphologie des plantes, et les effets des stress hydriques.

## III. Résultats

### III.1. Caractéristiques des souches bactériennes étudiées

#### III.1.1. Croissance des bactéries

La croissance de chaque souche bactérienne en milieu liquide a été suivie par densité optique à 595nm et modélisée par une courbe à partir d'au moins 3 répétitions biologiques (Fig. 2 et 3). Les paramètres déterminés à partir de cette modélisation ont révélé d'une part que la croissance des 4 souches de *Bradyrhizobium* est similaire, et d'autre part que ces souches ont une croissance plus lente que STM324 (Table 2). En effet, leur temps de latence est en moyenne 6 fois supérieur à celui de STM324. De plus, leur vitesse maximale de croissance en phase exponentielle est deux fois moins importante par rapport à STM324.

Afin de vérifier en fin d'expérimentation la présence de bactéries dans les milieux gélosés initialement inoculés, des carottes de gélose ont été prélevées puis étalées sur milieu semi-solide en boîte de Pétri. La présence de colonies bactériennes sur ces boîtes, quelques jours après l'étalement, a permis de confirmer la survie de toutes les souches bactériennes tout au long de la culture des plantules *in vitro*. Pour les boîtes coulées avec du milieu plante non inoculé, aucun des étalements n'a permis d'observer de colonies (données non montrées).

La croissance des bactéries a également été évaluée dans un milieu liquide contenant 13% de PEG afin de simuler un stress hydrique modéré. La  $DO_{595nm}$  mesurée dans ces

conditions a été comparée à celle mesurée en condition contrôle (milieu sans PEG) pour un même temps de culture (Fig. 4). La présence de PEG dans le milieu liquide a freiné la croissance de toutes les souches étudiées. Les *Bradyrhizobia* ont été les plus affectées avec une concentration bactérienne plus faible de 70% pour ORS3409 en présence de PEG. Au contraire, la concentration de STM196 et STM324 dans le milieu de culture liquide n'a été réduite que d'environ 40%.

### III.1.2. Capacité de production de composés auxiniques chez les souches bactériennes testées in vitro

L'auxine est reconnue comme ayant un effet stimulateur sur l'initiation de racines latérales ainsi que sur leur élongation. Par ce biais, cette hormone est susceptible d'avoir une influence sur la croissance des plantes. Il est donc intéressant de savoir si les souches bactériennes qui ont été testées dans cette étude sont capables de produire des composés auxiniques. Les souches bactériennes testées *in vitro* ont été cultivées en milieu liquide en présence de Tryptophane afin de tester leur capacité intrinsèque à sécréter des composés auxiniques à partir de cet acide aminé. Cette expérience a aussi été réalisée en ajoutant du PEG 13% afin de voir si le métabolisme bactérien et en particulier, la biosynthèse d'auxine, est affecté lors d'un stress hydrique modéré.

Globalement, la capacité de production de composés auxiniques par les souches ORS3258, ORS3409, ORS3410, ORS3411 et STM196 est très faible (Fig. 5). Des études antérieures (Contesto et al., 2010) ont montré que les niveaux de production d'auxine de STM196 (même niveau que cette expérimentation) étaient identiques à celui d'*Azospirillum jdp*-, une souche mutante prise comme référence comme souche non productrice d'auxine. En conclusion, nos résultats suggèrent que seule STM324 est capable d'avoir une production d'auxine : jusqu'à 0,1 ng/ufc, soit 24 fois plus que ORS3258.

Un autre résultat intéressant est que la présence de PEG occasionne des très faibles variations, même si elles sont significatives, des capacités de production d'auxine des bactéries peu productrices. La production d'auxine chez STM324 reste inchangée en présence de PEG. En conséquence, il peut être conclu que le PEG n'occasionne pas de modifications importantes dans les capacités de production d'auxine des bactéries sélectionnées dans cette étude.



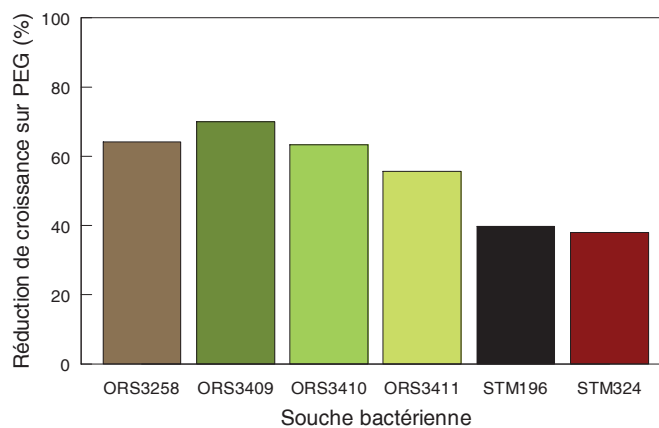


Fig. 4. Effet du PEG à 13% sur la croissance des souches bactériennes en milieu liquide.

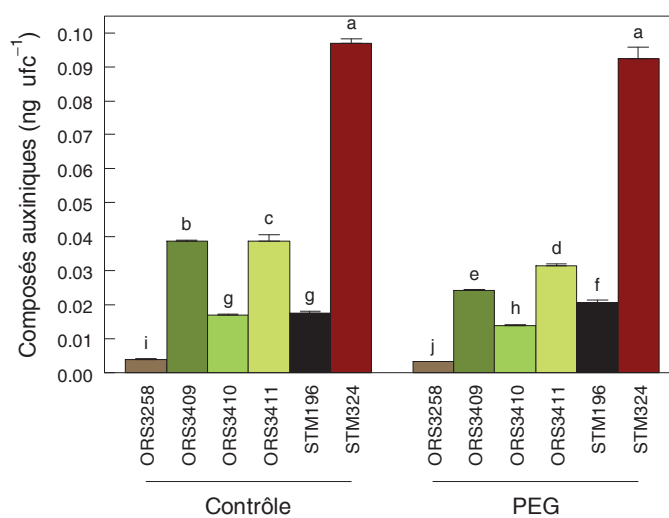


Fig. 5. Production de composés auxiniques par les souches bactériennes dans un milieu riche en Trp sans traitement (contrôle) ou contenant du PEG à 13%.

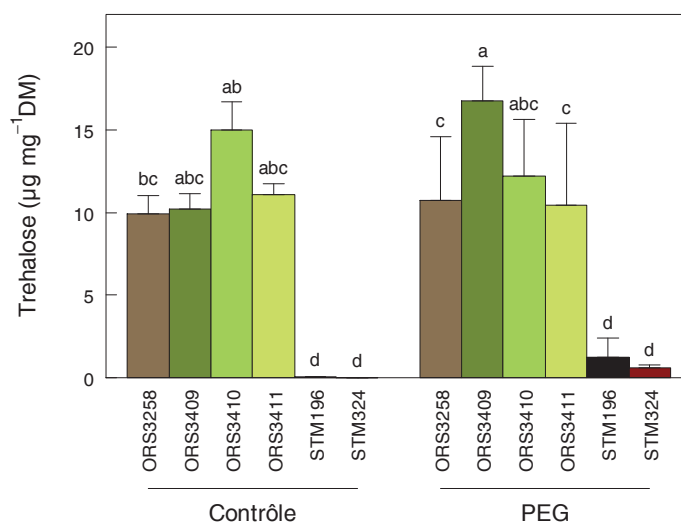


Fig. 6. Teneur en tréhalose mesuré par dosage enzymatique chez les souches bactériennes en conditions témoin (contrôle) et en milieu contenant du PEG à 13%.

### III.1.3. Teneur en tréhalose chez les souches bactériennes testées in vitro

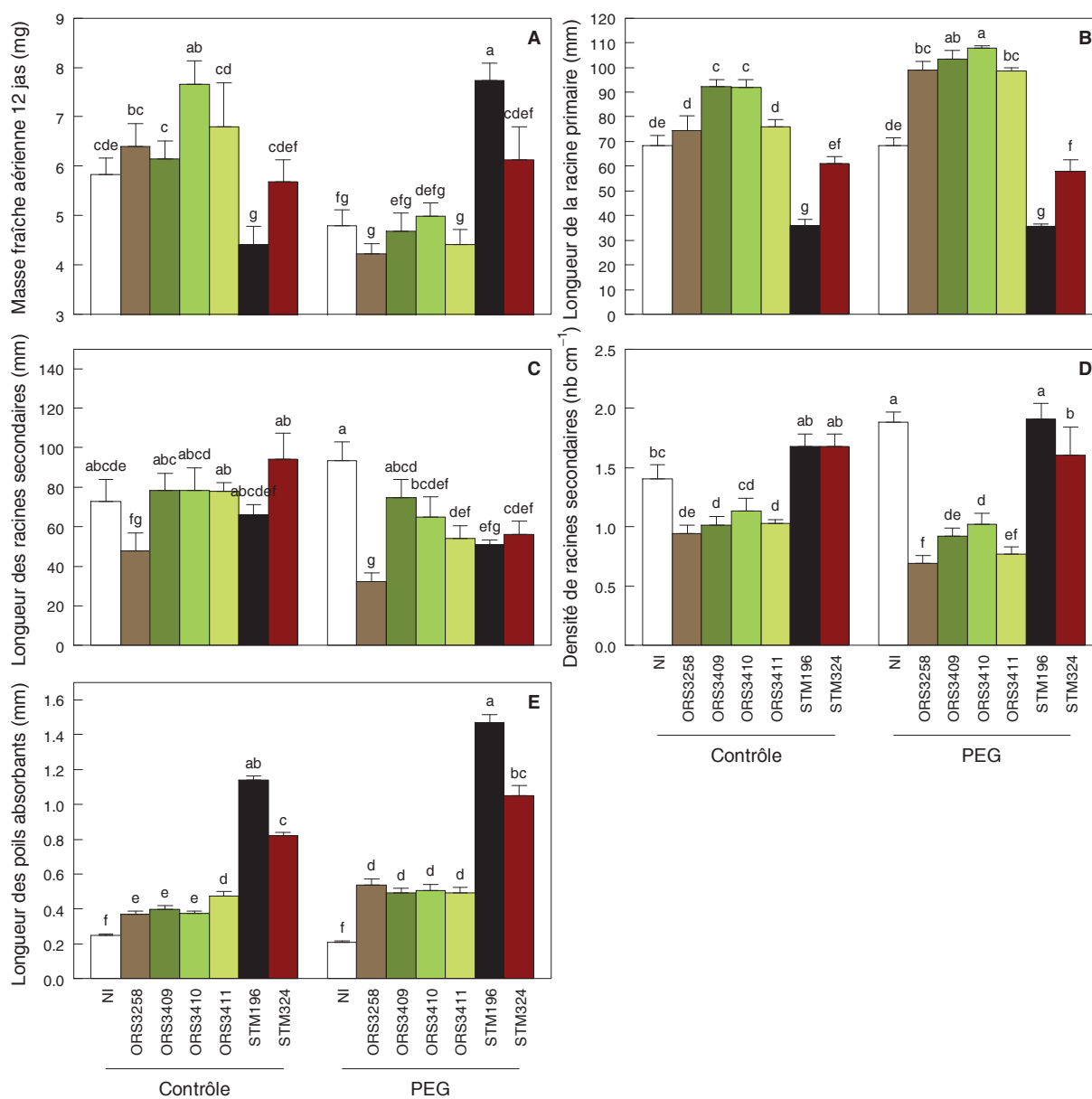
Des études ayant montrées que des bactéries transgéniques surproduisant du tréhalose étaient capables d'induire des résistances à la sécheresse sur les plantes inoculées (Rodriguez-Salazar *et al.*, 2009 ; Suarez *et al.*, 2008), il est intéressant d'évaluer la production de tréhalose par les bactéries. La teneur en tréhalose dans les cellules bactériennes a été déterminée par un dosage enzymatique dont le principe est basé sur le suivi de l'apparition de NADPH grâce à des mesures d'absorbance à 340nm (Annexe 1).

En condition contrôle, la teneur en tréhalose chez STM196 et STM324 a été détectée en proportions infimes (Fig. 6). Au contraire, cette teneur s'est révélée plus importante chez les souches de *Bradyrhizobium* (de l'ordre de 10  $\mu\text{g mg}^{-1}$ , pour ORS3409, ORS3410 et ORS3258, et jusqu'à 15  $\mu\text{g mg}^{-1}$  de matière sèche pour ORS3410).

La présence de 13% de PEG dans le milieu de culture liquide a entraîné une augmentation de la teneur en tréhalose chez STM196, STM324 (1,2 et 0,6  $\mu\text{g mg}^{-1}$  de matière sèche, respectivement). L'augmentation de la teneur en tréhalose pour les souches STM196 et STM324 est suffisamment importante pour envisager le fait qu'elle est utile pour la survie de la bactérie au stress hydrique. Par contre, les *Bradyrhizobium* (ORS3409 mis à part) semblent assez insensibles au PEG en ce qui concerne la production de tréhalose.

### III.2. Impact des différentes bactéries lors d'un stress modéré, *in vitro* en présence de polyéthylène glycol (PEG) sur le développement d'*Arabidopsis thaliana*

Pour étudier *in vitro* l'impact des 6 souches de bactéries sur la croissance d'*Arabidopsis*, des graines ont été directement semées sur des milieux inoculés. Pour chacune des souches la moitié des boîtes a été traitée afin d'obtenir un milieu à 13% de PEG. Des mesures de croissances (pesées) des parties aériennes ont été effectuées à 7 et 12 jours après semis. L'architecture racinaire et les poils racinaires ont été observés à 12 jours après semis. Enfin, la présence de bactéries vivantes dans le milieu de culture a été recherchée 15 jours après semis.



**Fig. 7. Caractérisation phénotypique 12 jours après semis (jas) des plantules d'*Arabidopsis thaliana* Col-0 cultivées in vitro avec ou sans PEG.**

A, Masse fraîche des parties aériennes. B, Longueur de la racine primaire. C, Longueur des racines secondaires. D, Densité des racines secondaires. E, Longueur des poils absorbants.

### III.2.1. Effet de l'application de PEG à 13% sur le milieu de culture non inoculé

Afin d'étudier l'effet d'un stress hydrique modéré *in vitro* chez *Arabidopsis*, un milieu contenant 13% de PEG a été préparé. Cette solution de PEG induit une diminution du potentiel hydrique du milieu de culture passant à environ -0.2Mpa. Cette concentration en PEG n'a pas eu d'impact sur le taux de germination. Sept jours après semis, la taille des parties aériennes n'est pas affectée par le PEG. Par contre, les racines primaires des plantes poussant sur le milieu PEG sont plus grandes (résultats non présentés). Ainsi, il semblerait qu'à ce stade de développement, la plante poussant sur PEG arrive à maintenir une croissance aérienne correcte en maximisant son système racinaire. 12 jours après semis, les effets de la présence de PEG dans le milieu commencent à apparaître: le PEG entraîne une diminution de 36% de la matière fraîche aérienne chez les plantes non inoculées (Fig. 7A) et ceci malgré le fait que la plante ait modifié son architecture racinaire permettant d'augmenter potentiellement la récupération d'eau (densité de racines secondaires significativement augmentée ; Fig. 7D). Par contre, le traitement au PEG n'induit pas de modification de la longueur de la racine primaire (Fig. 7B) ou de la longueur des poils absorbants (Fig. 7E).

### III.2.2. Effet des souches bactériennes sur le développement d'*Arabidopsis thaliana* cultivé *in vitro* en absence de PEG

Sept jours après semis, toutes les bactéries à l'exception de STM196 semblent avoir un effet positif sur la croissance d'*Arabidopsis*. Douze jours après semis, les effets des bactéries sur les *Arabidopsis* semblent s'atténuer. Sur milieu inoculé avec une souche de *Bradyrhizobium*, la matière fraîche aérienne a eu tendance à augmenter (Fig. 7A). Cette augmentation a même été significative en présence d'ORS3410 avec un gain de 66% de matière fraîche aérienne par rapport au témoin. Cette modification de croissance de l'appareil végétatif peut être associée à des modifications au niveau des racines. Les souches de *Bradyrhizobium* ont favorisé l'allongement de la racine primaire : celle des plantes inoculées par ORS3409 et ORS3410 a augmenté de 35% (Fig. 7B). En ce qui concerne la longueur des poils racinaires, celle-ci a été augmentée de 50 à 90% par rapport au témoin pour les milieux inoculés avec une souche de *Bradyrhizobium* (Fig. 7E). Par contre, la densité des racines secondaires chez les plantes inoculées avec une souche de *Bradyrhizobium* a été réduite par rapport au témoin (Fig. 7D).

Le cas de STM324 est différent puisqu'à 12 jns cette souche ne semble plus avoir d'effet significatif sur la croissance d'*Arabidopsis thaliana*. Pourtant cette bactérie modifie

profondément le système racinaire d'*Arabidopsis* avec des accroissements de la taille des poils absorbants d'un facteur 3 et un accroissement de la densité en racines latérales (Fig. 7).

Enfin STM196 a un effet très néfaste sur la croissance d'*Arabidopsis* dans cette expérience puisque la masse foliaire est moitié moindre de celle des plantes inoculées. Cette réduction de croissance des parties aériennes s'accompagne d'une baisse drastique de la longueur de la racine primaire, d'une augmentation de la taille des poils racinaires d'un facteur 4 et de l'augmentation de la densité en racines latérales (Fig. 7).

### III.2.3. Effet des souches bactériennes sur le développement d'*Arabidopsis thaliana* cultivé in vitro en présence de PEG (stress hydrique modéré)

En présence de PEG, la croissance des plantes inoculées par des *Bradyrhizobia* n'est pas significativement affectée par rapport aux plantes non inoculées. La croissance des plantes inoculées par ORS3258 et ORS3411 a tendance à être ralentie par rapport aux plantes témoins (Fig. 7A). Le traitement au PEG a tendance à accentuer les différences observées sans PEG, à savoir un allongement de la racine primaire (de l'ordre de 50% par rapport aux plantes non inoculées contre 35% au maximum en condition contrôle, Fig. 7B), une diminution de la densité des racines latérales (allant jusqu'à 57% sur le milieu inoculé avec ORS3258, Fig. 7D) et un accroissement de la taille des poils absorbants (Fig. 7E). Cette tendance est même significative pour ORS3258 et ORS3411.

Le PEG ne modifie pas le système racinaire (longueur racine primaire, densité des racines latérales, longueur poils absorbants) des plantes inoculées par STM324 (Fig. 7B,D,E). D'une manière remarquable, il est aussi à noter que les plantes inoculées par STM324 ne voient pas leur masse fraîche aérienne diminuée (Fig. 7A).

Comme pour STM324, le PEG ne change pas significativement les modifications du système racinaire des plantes occasionnées par une inoculation par STM196 (Fig. 7B,D,E). Par contre, STM196 a engendré un gain de matière fraîche de 164% par rapport au témoin, ce qui s'oppose à l'effet observé en condition contrôle (Fig. 7A). STM196 semble donc inhiber la croissance foliaire en « conditions normales » et la stimuler en condition de stress hydrique modéré.

### III.3. Impact de déficits hydriques contrastés sur la croissance d'*Arabidopsis thaliana* Col-0 cultivé en terre en présence de bactéries

#### III.3.1. Impact d'un stress hydrique modéré continu sur *Arabidopsis thaliana* cultivé en terre

L'écotype Col-0 d'*Arabidopsis thaliana* a été cultivé dans des conditions d'humidité du substrat optimales pour le substrat utilisé (35% HR<sub>sol</sub>) et en condition de déficit hydrique modéré (20% HR<sub>sol</sub>) appliqué tout au long de l'expérimentation.

La totalité des individus cultivés en déficit hydrique modéré continu ont survécu (non montré), contrairement aux individus ayant subi un stress hydrique ponctuel mais plus marqué (7%, voir ci-dessous). Le maintien d'une humidité relative du sol à 20% au cours de la croissance des plantes a clairement affecté la production de masse fraîche foliaire aussi bien chez les plantes cultivées dans une terre non inoculée que dans une terre inoculée avec une souche bactérienne (Fig. 8 et 9). Les plantes non inoculées ont perdu 70% de leur masse fraîche foliaire par rapport aux plantes cultivées sur une terre bien irriguée.

#### III.3.1. Impact d'un stress hydrique sévère ponctuel sur *Arabidopsis thaliana* cultivé en terre

Un stress plus sévère a été expérimenté au cours de la culture en terre d'*Arabidopsis thaliana* Col-0 : l'irrigation des pots a été stoppée à partir de l'apparition des deux premières feuilles et reprise qu'une fois l'humidité relative du substrat ayant atteint 7% afin de revenir à la condition initiale (35% HR sol).

Ce type de stress, plus marqué, n'a pas été toléré par l'ensemble de l'effectif sur lequel il a été appliqué, contrairement au stress modéré continu. Seulement 20% des plantes non inoculées ont survécu (Fig. 11).

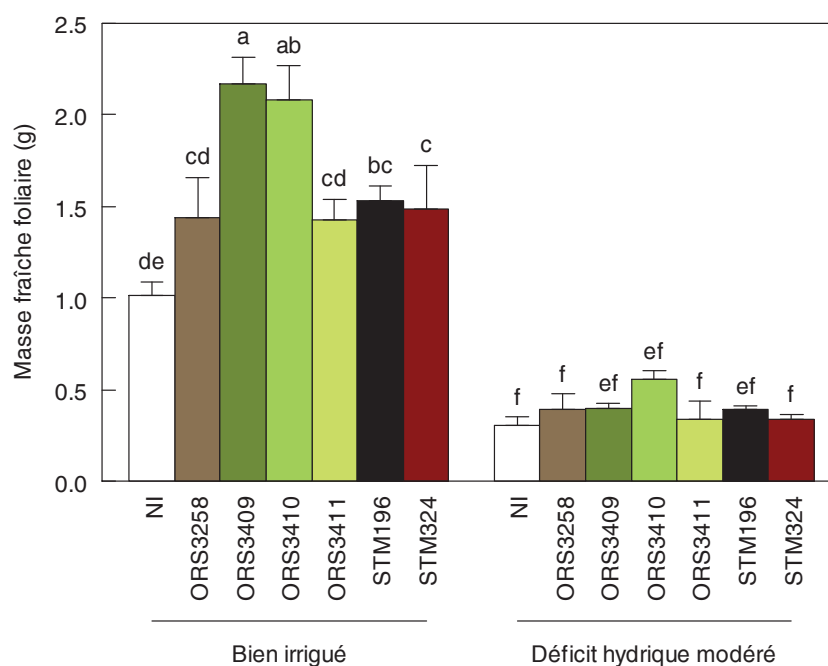
#### III.3.2. Impact des souches bactériennes sur *Arabidopsis thaliana* cultivé en terre

Parmi les bactéries testées en terre bien irriguée (35% HR<sub>sol</sub>), seules les souches ORS3258 et ORS3411 n'ont pas eu d'effet significatif sur la masse fraîche foliaire par rapport au témoin (Fig. 9). En revanche, les souches ORS3409 et ORS3410 ont doublé la quantité de masse



**Fig. 8.** Illustration de l'effet des stress hydriques testés en terre sur le développement de Col-0 à 36 jours après semis.

De gauche à droite : Condition contrôle : 35% d'humidité relative du sol ; condition de stress modéré : 20% d'humidité relative du sol ; condition de stress sévère: dessèchement du substrat jusqu'à 7%.



**Fig. 9.** Masse fraîche des feuilles de la plante au stade première fleur ouverte en conditions bien irrigué (35% humidité relative du sol) ou en déficit hydrique modéré (20% humidité relative du sol) et en conditions de substrat non inoculé (NI) ou inoculé par la souche bactérienne spécifiée.

fraîche par rapport aux plantes cultivées en absence de bactéries. Par ailleurs, il se dégage une tendance de la souche STM196 à augmenter la masse sèche du système racinaire (Fig. 10).

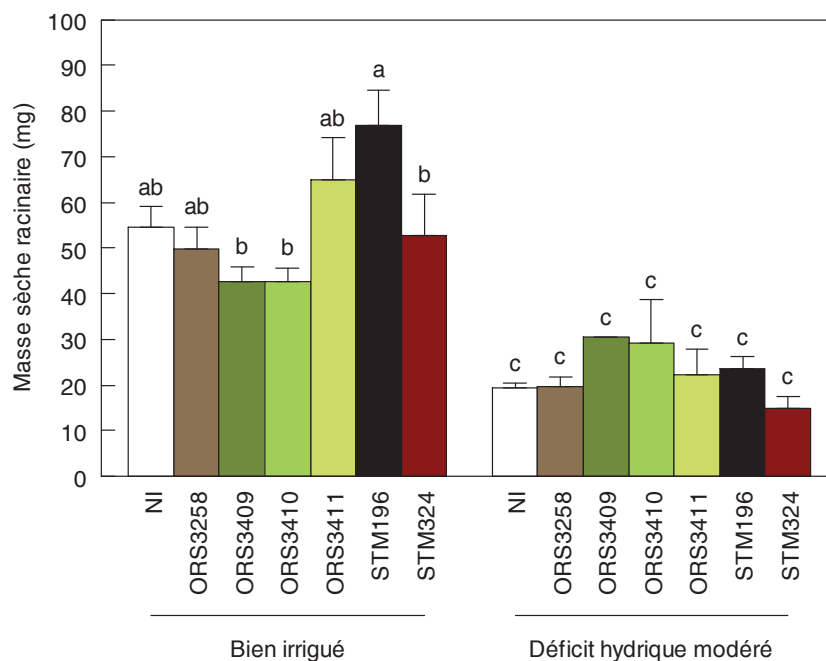
En condition de stress hydrique modéré continu, l'inoculation de bactéries dans le substrat de culture n'a pas eu d'effet significatif sur la masse fraîche des plantes cultivées (Fig. 9). Nous avons vu que la perte de masse fraîche induite par le stress hydrique modéré était de 70% en absence de bactéries. Cette perte a été plus conséquente pour les plantes cultivées dans un sol inoculé. Notamment, la souche ORS3409 a nettement réduit la masse fraîche avec des pertes dépassant les 80% de la valeur observée en condition d'irrigation optimale (Fig. 9). Au niveau de la rhizosphère, la perte de masse sèche racinaire est de l'ordre de 30% pour ORS3410 et ORS3411 tandis qu'elle est 2 fois plus conséquente pour les autres souches (Fig. 10).

En ce qui concerne le stress hydrique sévère, le taux de survie des plantes cultivées sur un substrat inoculé a toujours été supérieur à celui obtenu sur un sol non inoculé (Fig. 11). Ce taux a même avoisiné les 80% pour les plantes cultivées en présence de la souche ORS3411. La souche ORS3409 a nettement réduit la masse fraîche avec des pertes dépassant les 80% de la valeur observée en condition d'irrigation optimale.

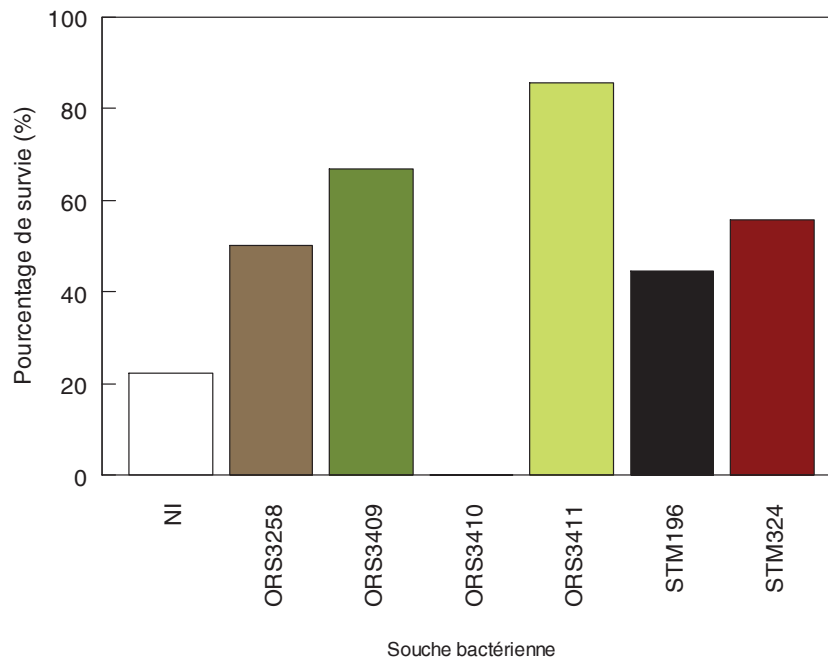
## IV. Discussion

Certaines souches bactériennes présentes dans le sol peuvent interagir par divers mécanismes avec la rhizosphère des plantes et ainsi stimuler leur croissance. Ces bactéries sont alors considérées comme des PGPR (Plant Growth Promoting Bacteria) de par leurs effets bénéfiques pour la plante. Les bactéries du genre *Bradyrhizobium*, partenaires symbiotiques des Légumineuses, partagent des mécanismes communs aux PGPR : production de phytohormones et de sidérophores, par exemple. La souche ORS278 de *Bradyrhizobium* sp. a la capacité de coloniser la rhizosphère du riz *Oryza breviligulata* et d'établir une relation endophytique bénéfique à la croissance de cette plante non Légumineuse (Chaintreuil *et al.*, 2000). Au cours de cette étude, l'impact de 6 souches bactériennes présélectionnées à partir de la souchothèque du LSTM, a été testé sur le développement d'*Arabidopsis thaliana* Col-0 en conditions optimales et de stress hydrique. Des analyses phénotypiques ont été effectuées sur des plantes cultivées *in vitro* et en terre afin de déterminer l'effet de chaque souche sur le développement de la plante. *In vitro*, un stress hydrique modéré a été simulé en ajoutant une solution de Polyéthylène glycol dans le milieu de culture. En terre, un stress modéré continu





**Fig. 10.** Masse sèche du système racinaire de la plante au stade première fleur ouverte en conditions bien irrigué (35% humidité relative du sol) ou en déficit hydrique modéré (20% humidité relative du sol) et en conditions de substrat non inoculé (NI) ou inoculé par la souche bactérienne spécifiée.



**Fig. 11.** Pourcentage de survie des plantes d'*Arabidopsis thaliana* Col-0 cultivées en conditions de stress hydrique sévère (7% d'humidité relative du sol suivi d'une ré-irrigation jusqu'au niveau bien irrigué (35%)) en conditions de substrat non inoculé (NI) ou inoculé par la souche bactérienne spécifiée.

et un stress sévère ponctuel ont pu être appliqués en contrôlant l'humidité relative du substrat de culture.

#### IV.1. Mise en évidence d'une interaction entre les bactéries testées et *Arabidopsis thaliana*

Globalement, dans des conditions optimales de culture, toutes les bactéries testées ont eu un effet positif sur la croissance des parties aériennes des plantes. Ce résultat confirme le statut de PGPR de *Pseudomonas fluorescens* et de *Phyllobacterium brassicacearum* STM196. Ceci démontre également que des bactéries isolées à partir de nodules formés sur une Légumineuse peuvent établir une relation bénéfique avec une plante phylogénétiquement éloignée de leur plante hôte. De plus, il confirme le fait que les effets PGPR bactériens ne sont pas spécifiques d'une seule plante et qu'ils peuvent être étudiés sur des espèces modèles telles qu'*Arabidopsis thaliana*. Les résultats obtenus en terre confirment l'intérêt de l'utilisation d'un sol ionisé qui permet de s'affranchir des problèmes de compétition entre bactéries présentes dans des sols non stérilisés pour la colonisation de la rhizosphère.

Le stress hydrique modéré appliqué en terre a affecté significativement la croissance des plantes sans avoir d'effet létal. Lorsqu'un stress modéré est appliqué, les bactéries testées n'améliorent pas la croissance de manière significative. L'application d'un stress plus fort avec une chute d'humidité relative du sol jusqu'à 7%, n'est pas toléré par tous les effectifs de plantes. Les bactéries étudiées ont joué un rôle important dans la résistance à ce stress hydrique sévère. Un autre aspect du rôle des bactéries PGPR a ainsi été mis en évidence : la résistance de la plante en cas de stress abiotique.

Au final, le cas d'ORS3410 mis à part, l'inoculation par les différentes bactéries provoque des effets bénéfiques similaires. Les expériences faites sur les bactéries isolées suggèrent néanmoins que les mécanismes d'action responsables de ces différents effets bénéfiques sont très différents d'une bactérie à l'autre.

#### IV.2. Des bactéries du genre *Bradyrhizobium* stimulent la croissance d'*Arabidopsis thaliana*

Le résultat le plus spectaculaire est probablement l'augmentation très importante de la survie d'*Arabidopsis thaliana* en condition de stress sévère lorsque les plantes sont inoculées par toutes les bactéries, à l'exception d'ORS3410. Il est remarquable de constater, qu'au sein de *Bradyrhizobia* phylogénétiquement très proches (ORS3409, ORS3410 et ORS3411

appartiennent au groupe VI), isolées sur la même plante, et vivant dans des conditions climatiques comparables (climat aride), on trouve des effets sur la survie des plantes très différents. Ces souches étant en cours de séquençage, il serait intéressant d'effectuer une analyse de génomique comparative pour identifier les différences entre le génome d'ORS3410 et celui des autres souches. Les résultats obtenus *in vitro* suggèrent que cette différence n'est pas liée à la biosynthèse d'auxine et de tréhalose, ni au mode de croissance de ces souches, ni aux capacités de survie en cas de stress hydriques modérés.

Le mécanisme d'action des *Bradyrhizobia* testées est probablement très différents de celui de STM324 (voir ci-dessous). Une grosse différence vient des capacités très faibles de synthèse d'auxine par les *Bradyrhizobia*. Cette différence se ressent au niveau de l'observation du système racinaire *in vitro* qui n'est pas compatible avec la sécrétion d'auxine par la bactérie. Ces résultats ne sont probablement pas généralisables à toutes les *Bradyrhizobia*. En effet, une étude effectuée sur *Bradyrhizobium japonicum* a déjà révélé la capacité de certaines souches à produire de l'AIA (Antoun *et al.*, 1998).

Les souches de *Bradyrhizobium* testées dans cette étude se distinguent des autres bactéries testées, notamment du fait de leur grande production de tréhalose. Ce résultat est en accord avec les données de séquençage sur ces souches qui révèlent la présence de nombreux gènes de biosynthèse du tréhalose (Le Quéré, non publié). En revanche, il ne semble pas y avoir d'effet type sur la production de tréhalose. Le tréhalose et son intermédiaire, le tréhalose 6P ayant des effets marqués sur la croissance des plantes et leur capacité à augmenter la résistance à un déficit hydrique, il est tout à fait envisageable que les effets bénéfiques observés sur *Arabidopsis thaliana* soient la résultante de cette synthèse importante de tréhalose.

#### IV.3. Effet PGPR de *Pseudomonas fluorescens* STM324 chez *Arabidopsis thaliana* : un effet auxine dépendant ?

Les résultats obtenus révèlent que la capacité de STM324 à synthétiser de l'auxine semble être corrélée avec les effets observés *in vitro* sur le développement du système racinaire d'*Arabidopsis thaliana* (raccourcissement de la racine primaire et allongement des poils racinaires) d'une part et sur la protection conféré face à un stress hydrique d'autre part.

Il a été démontré que l'inoculation de graines de radis avec une souche de *Pseudomonas fluorescens* génératrice d'auxine augmente la masse fraîche racinaire (Kamilova *et al.*, 2006). Les composés auxiniques sécrétés par certaines bactéries dans le sol

sont produits à partir d'un acide aminé (Tryptophane) contenu dans les exsudats racinaires de la plante. La synthèse de tels composés dépend donc de la concentration en Trp dans les exsudats racinaires de la plante. Par exemple, cette concentration est 9 fois plus conséquente dans les exsudats racinaires du radis que dans ceux du concombre ou de la tomate (Kamilova *et al.*, 2006). Il serait intéressant d'établir un protocole permettant de mesurer cette concentration dans les exsudats racinaires d'*Arabidopsis thaliana* à partir de carottes de milieu gélosé prélevées à proximité des racines de la plante. La concentration de cet acide aminé, qui a la caractéristique d'absorber la lumière à une longueur d'onde de 280 nm, peut être déterminée par densité optique. Un effet auxinique sur la rhizosphère d'une plante dans le cadre d'une interaction plante/bactérie dépend aussi bien des exsudats racinaires que de la capacité de la bactérie à produire cette hormone. Il faut également prendre en compte le fait que l'auxine agit de manière dose-dépendante sur la rhizosphère d'*Arabidopsis thaliana*. Ainsi, les effets sur le développement racinaire peuvent être opposés selon la quantité d'AIA sécrétée par les bactéries dans le milieu.

Mis à part son effet sur le système racinaire secondaire, STM324 a conféré à *Arabidopsis* une protection face au stress hydrique. Une étude récente a démontré que la production d'AIA par *Pseudomonas* sp. est corrélée à la tolérance au stress hydrique du maïs (Marulanda *et al.*, 2009). L'AIA sécrétée par les bactéries pourrait stimuler le développement du système racinaire de la plante qui maximise du même coup ses capacités d'absorption d'eau et de résistance à la sécheresse. Le faible potentiel en eau du milieu est compensé par une augmentation de la rhizosphère et n'a pas de répercussion sur la production de matière fraîche foliaire.

#### IV.4. *Phyllobacterium brassicacearum* STM6 : une PGPR référence d'*Arabidopsis thaliana*

Tout comme STM324, *Phyllobacterium brassicacearum* STM196 est une souche à croissance rapide. Sa capacité à produire des composés auxiniques est cependant très faible ainsi que sa production de tréhalose. Des expériences montrent cependant que STM196 est capable de modifier les voies de signalisation de l'auxine (Contesto *et al.*, 2010) dans les racines et qu'elle provoque une augmentation de la teneur en tréhalose et en tréhalose-6-P dans la plante (Tasselli, 2007). Les résultats obtenus en terre révèlent un effet PGPR de cette souche en condition d'irrigation optimale. Une augmentation significative de la masse sèche du système

racinaire peut expliquer une optimisation d'absorption d'eau permettant d'augmenter la matière fraîche dans les feuilles.

Afin de progresser dans la compréhension des mécanismes des rhizobactéries influençant la croissance d'*Arabidopsis thaliana*, il serait intéressant de faire des expérimentations supplémentaires visant notamment à mesurer la production d'éthylène des plantes inoculées, de tester les capacités de production d'autres phytohormones telles que les cytokinines. Aussi, il serait intéressant de déterminer si les bactéries testées sont capables de fixer l'azote atmosphérique librement ou de fournir de l'ammonium à la plante dans nos conditions expérimentales.

#### IV.5. Est-il possible de sélectionner des bactéries avec un système en terre et/ou *in vitro* ?

Chacun des deux systèmes présentent donc des avantages et des inconvénients. La culture de plantes *in vitro* permet tout d'abord de pouvoir réaliser des expériences sur des temps courts (de l'ordre de 12 jours), et de pouvoir disposer d'un effectif conséquent. Le système *in vitro* permet également d'accéder à des données d'architecture racinaire, plus difficilement accessibles en terre. Cependant, le temps de culture en boîte de Pétri est restreint. Il n'est donc pas possible d'analyser des réponses développées par la plante sur tout le long de son cycle de vie. Un mode de culture en terre permet donc d'analyser des dynamiques de croissance et de pouvoir réaliser des mesures d'écophysiologie comme notamment des mesures de transpiration et de photosynthèse (résultats non montrées).

Par contre, il semble plus difficile de comparer les effets du PEG avec un stress hydrique réalisé en terre. En effet, l'utilisation du PEG pour limiter la disponibilité en eau peut être problématique et créer des biais. L'ajout de PEG dans le milieu de culture augmente la viscosité de la gélose et peut empêcher la diffusion de l'oxygène et expliquer le fait que les plantes puissent souffrir d'hypoxie (Verslues *et al.*, 1998). De plus, l'ajout de PEG peut être néfaste pour la croissance bactérienne, ou au contraire peut servir de nutriment pour certaines bactéries. Il a été montré que certaines bactéries dont le genre *Pseudomonas* sont capables de dégrader le PEG et de l'utiliser comme source de carbone (Pan *et al.*, 2007). Dans notre cas, il semblerait que les concentrations de PEG appliquées provoquent un ralentissement de croissance des bactéries (Figure 4).

En plus de fournir des indications précieuses sur l'architecture racinaire, difficilement accessibles par des expériences en terre, les expériences *in vitro* ont eu une bonne valeur

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prédictive sur les effets des bactéries en conditions de bonne alimentation hydrique et de stress modéré. Les précédentes expérimentations avaient montré que STM196 était capable de stimuler la croissance des plantes. Le fait que ce résultat n'ait pas été reproduit suggère qu'il est dangereux de baser un crible sur une expérimentation unique et qu'il faut envisager de réaliser des répétitions biologiques.

Ces deux méthodes expérimentales sont complémentaires et permettent de disposer de données utiles à la sélection de bactéries.

## V. Conclusion

L'étude phénotypique du comportement d'*Arabidopsis thaliana*, plante modèle par excellence, en présence de souches bactériennes, permet de refléter les mécanismes mis en jeu dans l'interaction plante/bactérie. Cette étude ouvre des perspectives d'étude génétique sur les mécanismes d'interaction plante/bactérie *via* l'utilisation de mutants bactériens et/ou de plante. L'utilisation d'une plante modèle permet de transposer plus facilement l'impact d'une bactérie chez sa plante hôte dont le génome n'a pas été totalement séquencé. La compréhension des relations entre bactéries et plante hôte offre aussi l'opportunité de sélectionner des souches bactériennes à tester en champ pour leur impact sur la productivité et la résistance à la sécheresse de plantes largement cultivées dans le monde.

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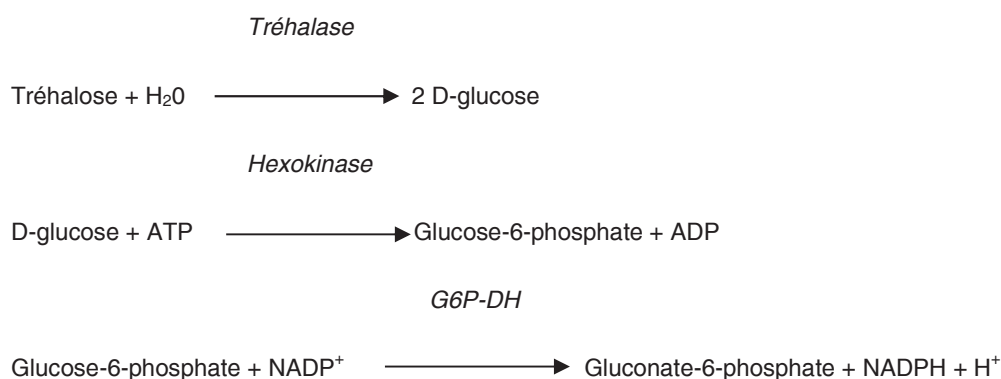
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## Annexes

### ANNEXE 1. Principe du dosage enzymatique du tréhalose

**A :** L'hydrolyse du tréhalose par la tréhalase conduit à la formation de 2 molécules de D-glucose. En présence d'ATP, l'Hexokinase permet la formation de Glucose-6-phosphate à son tour transformé en Gluconate-6-phosphate en présence de  $\text{NADP}^+$ . La quantité de NADPH formée est stœchiométrique avec la quantité de D-glucose et donc 2 fois plus importante que la quantité de tréhalose. L'augmentation de l'absorbance dans le mélange réactionnel après ajout de la tréhalase est due à la formation de NADPH qui absorbe la lumière à 340nm. **B :** La concentration en tréhalose dans le surnageant dosé a été calculé en fonction du volume réactionnel final  $V$  (1,27 mL), de la masse moléculaire du tréhalose  $MM$  (342,3  $\text{g}\cdot\text{mol}^{-1}$ ), du coefficient d'extinction molaire du NADPH à 340nm  $\epsilon$  (6300  $\text{l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ ), du volume de surnageant  $v$  (0,01mL), du facteur de dilution  $F$  (10), du nombre de molécules de D-Glucose libérées pour chaque molécule de tréhalose hydrolysée et de la différence d'absorbance à 340nm entre l'absorbance du mélange réactionnel après l'ajout de tréhalase et avant l'ajout de tréhalase.

#### A. Hydrolyse du tréhalose



#### B. Calcul de la concentration en tréhalose

$$C = \frac{V \cdot M \cdot F}{\epsilon \cdot v \cdot 2} \cdot \Delta A$$

$$C = \frac{1,27 \cdot 342,3 \cdot 10}{6300 \cdot 0,01 \cdot 2} \cdot \Delta A$$

$$C = 0,345 \cdot \Delta A$$



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## ***Annexe 2***

***Arabidopsis growth under  
prolonged high temperature and  
water deficit: independent or  
interactive effects?***

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## ***Arabidopsis* growth under prolonged high temperature and water deficit: independent or interactive effects?**

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### **ABSTRACT**

**High temperature (HT) and water deficit (WD) are frequent environmental constraints restricting plant growth and productivity. These stresses often occur simultaneously in the field, but little is known about their combined impacts on plant growth, development and physiology. We evaluated the responses of 10 *Arabidopsis thaliana* natural accessions to prolonged elevated air temperature (30 °C) and soil WD applied separately or in combination. Plant growth was significantly reduced under both stresses and their combination was even more detrimental to plant performance. The effects of the two stresses were globally additive, but some traits responded specifically to one but not the other stress. Root allocation increased in response to WD, while reproductive allocation, hyponasty and specific leaf area increased under HT. All the traits that varied in response to combined stresses also responded to at least one of them. Tolerance to WD was higher in small-sized accessions under control temperature and HT and in accessions with high biomass allocation to root under control conditions. Accessions that originate from sites with higher temperature have less stomatal density and allocate less biomass to the roots when cultivated under HT. Independence and interaction between stresses as well as the relationships between traits and stress responses are discussed.**

*Key-words:* *Arabidopsis thaliana*; biomass allocation; hyponasty; leaf morphology; multistress; phenology; stomatal density.

### **INTRODUCTION**

High temperature (HT) and water deficit (WD) are two important environmental constraints restricting plant growth and productivity in many areas of the world (Boyer 1982; Ciais *et al.* 2005). Global climate change will presumably increase the occurrence and extend the distribution of these constraints, leading to further reduction of productivity and shifts in biodiversity (Chaves *et al.* 2002; Lobell & Asner 2003; Porter 2005; Thuiller *et al.* 2005; IPCC 2007). The two stresses often occur simultaneously in the field, but little is known about their combined effects on plant

growth, development and physiology (Machado & Paulsen 2001; Zhang *et al.* 2008).

Different mechanisms have been identified as ensuring plant survival and growth under elevated temperatures or water shortage. They include long-term evolutionary phenological and morphological adaptations and short-term avoidance or acclimation mechanisms. Even moderate increases in air temperature (Lafta & Lorenzen 1995; Loveys *et al.* 2002) or decreases in soil water availability (Passioura 1996) are responsible for impaired plant growth. Many elementary biological processes and morphological traits underlying plant growth are sensitive to temperature, and their responses repeatedly resemble a bell-shaped curve. As temperature rises above a particular threshold, processes such as net photosynthetic rate are negatively affected (Körner 2006; Sage & Kubien 2007; Parent *et al.* 2010), ultimately leading to a decline in plant performance. Temperature is also the main determinant of plant phenology (Ritchie & NeSmith 1991), and moderate increases in air temperature generally accelerate the rate of developmental processes leading to early flowering in most wild and cultivated species (Johnson & Thornley 1985). Whereas the effects of WD on phenology remain elusive, delayed timing of reproduction is often observed in crop species (McMaster *et al.* 2009). The effects of these stresses also depend on the phenological stage at which they occur (Prasad, Staggenborg & Ristic 2008). For instance, HT has greater impacts on seed yield during the reproductive phase (Jenks & Wood 2010). Therefore, accelerated reproduction in response to HT is generally viewed as an escape mechanism.

HT and WD have contrasted effects on patterns of biomass allocation to organs and tissues. For instance, allocation to roots rapidly increases in response to moderate soil WD (Boyer 1985), whereas leaf relative water content and specific leaf area (SLA) decline in plants subjected to water stress (Poorter *et al.* 2009). Leaf structure is also affected by temperature, but, in contrast to WD, higher temperature often leads to the production of thinner leaves with higher SLA (Boese & Huner 1990; Loveys *et al.* 2002; Luomala *et al.* 2005; Poorter *et al.* 2009). These morphological changes are accompanied by changes in leaf anatomy. Leaves developed under WD have generally smaller cells in the parenchyma and the epidermis (Lecoeur *et al.* 1995) and

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higher stomatal density (Aubert *et al.* 2010; Tisne *et al.* 2010). Wahid *et al.* (2007) reported similar effects of HT and WD on cell density, but limited data are available on changes in leaf anatomy in response to HT.

The effects of WD, particularly osmotic stresses or watering deprivation, and HT, particularly short periods of acute heat stress, have been mostly analysed separately. There is, however, strong evidence that HT and WD interact to influence plant functioning (Rizhsky, Liang & Mittler 2002; Rizhsky *et al.* 2004). For instance, WD induces stomatal closure and reduces transpiration fluxes (Hsaio 1973). This in turn can cause an increase in leaf temperature by reducing transpirational cooling (Cook, Dixon & Leopold 1964), and potentially enhances plant susceptibility to higher air temperature. Increase in leaf temperature can also raise plant water loss through transpiration (Lafta & Lorenzen 1995), and decrease root growth (Kuroyanagi & Paulsen 1988), thus increasing plant susceptibility to water shortage. By contrast, changes in leaf orientation in response to elevated temperature (Fu & Ehleringer 1989) such as hyponasty (Koini *et al.* 2009; Van Zanten *et al.* 2009) modify the leaf energy balance and could contribute to water saving by limiting rises in leaf temperature and evaporative demand. Hyponasty could also increase water consumption if associated with increased transpiration. Lastly, effects of HT on growth could lead to reduced leaf area, limiting plant water losses and thus mitigating the effects of WD.

In the face of the multiplicity of interacting, sometimes opposite effects between these two stresses, it appears difficult to predict plant responses to combined HT and WD. The aim of this study was therefore to evaluate the responses to both isolated and combined HT and WD in natural accessions of the model plant *Arabidopsis thaliana*. The following questions were addressed: (1) How do HT and WD interact on traits related to plant growth, morphology and development and to what extent do their combined effects differ from those of isolated stresses? (2) Is the variability of responses to isolated and combined HT and WD related to the climatic conditions at the accessions collection sites? (3) To what extent are these responses related to trait values exhibited in control conditions? A set of 10 *Arabidopsis* accessions spanning nearly the entirety of the latitudinal range of this species was selected to identify common responses and explore the natural variation of *Arabidopsis* tolerance to both stresses. Controlled environmental conditions were applied in full factorial experiments and maintained constant from the seedling to the reproductive stage. Control air temperature (CT) was set to 20 °C, as in most experimental studies (Balasubramanian *et al.* 2006; Saidi, Finka & Goloubinoff 2011), whereas HT was set to 30 °C. This HT level has been identified to be the basal thermotolerance, that is the highest temperature tolerated by a plant that has never encountered previous HT, of the *Arabidopsis* accession Col-0 (Ludwig-Muller, Krishna & Forreiter 2000). Soil WD was maintained constant at a level previously shown to significantly decrease leaf water potential and impair plant growth, resulting in reduced plant size of Col-0 by half (Aguirrezábal *et al.* 2006).

## MATERIALS AND METHODS

### Plant material and growth conditions

Ten accessions of *A. thaliana* were grown in one to three independent experiments depending on the accession (Table 1). Seeds of all genotypes were stored at 4 °C in the dark ensuring stratification. Five seeds from each genotype were directly sown at the soil surface in 225 mL culture pots filled with a mixture (1:1, v : v) of loamy soil and organic compost (Neuhaus N2). Pots were damped with sprayed deionized water three times a day and placed in two controlled growth chambers in darkness (20 °C, 65% air relative humidity) until germination. After germination, plants were cultivated with a daily cycle of 12 h light supplied from a bank of HQi lamps which provided 175  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD) at plant height.

Soil WD and HT treatments were applied to half of the pots after emergence of the first two true leaves (stage 1.02 in Boyes *et al.* 2001) ensuring a good establishment of the seedlings. In the first growth chamber, CT was set to 20/17 °C day/night, while HT treatment was set to 30/25 °C in the second one. Air relative humidity was adjusted to 65% under CT and 85% under HT in order to maintain equal water vapour pressure deficit (VPD) at 0.9 kPa. This was set up in order to avoid the confounding effect of temperature on transpiration through increased VPD. Soil water content was controlled before sowing to estimate the amount of dry soil and water in each pot. Subsequent changes in pot weight were due to changes in water status. Soil water content was maintained at 0.35 and 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil with a modified one-tenth strength Hoagland solution (Hoagland & Arnon 1950) in the well-watered (WW) and WD treatments, respectively. The field capacity of the substrate was 0.78 g H<sub>2</sub>O g<sup>-1</sup> dry soil (Granier *et al.* 2006); therefore, the WW and WD treatments represented 45 and 25% of the soil field capacity, respectively. Pot weight was precisely adjusted to reach the target soil water content by weighing and watering each individual pot every Monday, Wednesday and Friday. Other days, a standard volume of nutrient solution amounting to the mean volume of previously weighed water applications for each treatment was added to the plants without weighing the pots.

Three consecutive experiments were carried out following the same experimental procedure (see Table 1). In experiments 1 and 2, only one plant per pot was maintained until first silique shattering, while one to three plants were maintained until inflorescence emergence in experiment 3 for photosynthesis measurements and abscisic acid (ABA) content determination.

### Measurement of plant traits

During the course of plant development, the following stages were scored: germination, cotyledons fully opened, two rosette leaves >1 mm, inflorescence emergence, first flower open and first silique shattered (stages 0.7, 1.0, 1.02, 5.01, 6.00 and 8.00 of Boyes *et al.* (2001), respectively). Leaf

**Table 1.** Origin of the accessions studied and climate at the collection sites

Accession	Latitude (°N)	Longitude (°E)	Country	Mean autumn-spring precipitations (mm)	Mean autumn-spring temperature (°C)	Diurnal temperature range	Relative humidity	Exp. 1	Exp. 2	Exp. 3
Cvi-0	15	-23.4	Cape Verde Island	0.36	21.8	5.52	74.4		X	X
Mtr-0	32.6	22.8	Libya	52.2	15.2	7.57	62.0		X	X
Ct-1	37	15	Italy	61.19	12.3	8.73	73.5		X	X
Sha	38	68	Tadjikistan	53.1	8.2	13.16	59.5	X	X	X
Bay-0	49		Germany	29	2.99	8.58	77.0	X		
An-1	51.2	4.4	Belgium	64.2	5.96	7.74	82.2		X	X
Col-0	53	10	Poland	52.9	3.96	7.59	86.3	X	X	X
Ler	53	16	Poland	38.2	2.6	7.63	83.1	X	X	X
Lc-0	58	-5	UK	161.0	4.1	5.83	88.5		X	X
Est-1	59	28	Russia	48.7	-2.1	7.68	84.3		X	X

X indicates the experiments in which accessions were studied.

number was determined for each plant at each precise adjustment of soil water content, that is three times a week, only in experiments 2 and 3.

### Dynamics of leaf production

For each plant in experiment 2, a sigmoid curve was fitted to the relationship between total number of rosette leaves ( $LN$ ) and time from stage 1.02 to stage 8.00 by the following four-parameter logistic model:

$$LN = \frac{a}{1 + e\left(-\frac{(d-d_0)}{b}\right)} \quad (1)$$

where  $d$  is the number of days after stage 1.02,  $a$  is the maximum vegetative leaf number,  $d_0$  is the time when  $a/2$  leaves have developed and  $b$  is the inverse of slope factor which refers to the steepness of the curve, and is thus a parameter related to the maximum rate of leaf production. In order to standardize between genotypes, we used an estimate of leaf production duration (days) as  $d_0 - b \ln(0.05/0.95)$ , that is the time period for vegetative leaf number to increase from 5 to 95% maximum number. The maximum rate of leaf production ( $R_{\max}$ , leaf  $d^{-1}$ ) was calculated from the first derivative of the logistic model at  $d_0$  as  $R_{\max} = a/(4b)$ .

In experiment 3, since leaf emergence rate is maximal and nearly constant between stage 1.02 and stage 5.01,  $R_{\max}$  was fairly well estimated by the slope of the relationship between  $LN$  and time during this period.  $R_{\max}$  varied across genotypes and treatments with highly reproducible results between experiments ( $r = 0.85$ ,  $P < 0.001$ ). Most of the plants survived the HT and WD treatments, and reached the reproductive stage. Only a few plants did not survive the combined HT  $\times$  WD treatment.

### Whole plant and leaf traits

In experiment 2, 20 d after germination, tip height, total length and blade length of the youngest fully expanded leaf were measured on each plant with a digital calliper as described in Hopkins, Schmitt & Stinchcombe (2008). At this time, plants had six to 14 leaves depending on the genotype, and inflorescence had not emerged. Measurements were taken in randomized order between 2 and 4 h after lights went on in the chambers to avoid any effects associated with time of the day like endogenous rhythms. The proportion of leaf composed of blade was estimated by the blade ratio, the blade length divided by total leaf length. Leaf insertion angle (degree) was calculated as  $\theta = \arcsine(\text{leaf tip height/leaf length})$ .

Plants were harvested at stage 8.00, in the morning and after irrigation. Rosettes were cut, inflorescences were detached from the rosettes and their fresh weights (FWs) (milligrams) were determined immediately. Leaf blades were separated from the rosette, and FWs of the sixth and ninth leaves were determined. Mean leaf thickness (LT) of



these two leaves was determined with a linear variable displacement transducer (Solartron) connected to a multimeter and previously calibrated with 5  $\mu\text{m}$  accuracy. Depending on the size of the leaf, LT was measured on 6 to 10 points per leaf blade, avoiding the mid-vein. All blades were then stuck on a sheet of paper, arranged by order of emergence on the rosette, and the sheet of paper was scanned for area measurements. Additionally, a transparent imprint of the adaxial epidermis of the sixth leaf was obtained by drying off a varnish coat spread on the surface of the leaf. Imprint was peeled off and then stuck on microscope slides with one-sided adhesive for further measurements. Roots were carefully extracted from the soil and gently washed in deionized water. Leaf blades, petioles, reproductive structures and roots were then separately oven-dried at 65 °C for at least 3 d, and dry masses were determined. Rosette area ( $\text{cm}^2$ ) was determined as the sum of individual leaf blade areas measured on the scans with an image analysis software (Bioscan-Optimas 4.10, Edmond, WA, USA). From these measurements, leaf dry matter content (LDMC, the ratio of dry mass to fresh mass,  $\text{mg g}^{-1}$ ) and SLA (the ratio of leaf area to leaf dry mass,  $\text{m}^2 \text{kg}^{-1}$ ) were calculated at the rosette and leaf (for leaves 6 and 9) levels. Biomass allocation was assessed by the ratios of above-ground vegetative, reproductive and below-ground dry masses to total plant dry mass. Root-to-shoot ratio was calculated as the ratio of root to vegetative above-ground masses.

### Leaf epidermal anatomy

Epidermal imprints of the sixth leaves were placed under a microscope (Leitz DM RB; Leica, Wetzlar, Germany) coupled to an image analyser. Mean cell and stomatal densities were determined by counting the number of cells and stomata in two 0.12  $\text{mm}^2$  zones in the middle part of the leaf blade distributed on both sides of the mid-vein halfway from the margins. Stomatal index was calculated as  $100 \times \text{stomatal number} / (\text{stomatal number} + \text{stomatal number} \times 2 + \text{epidermal cell number})$ .

### Net photosynthetic rate

Net photosynthetic rate was measured using a single leaf chamber designed for *Arabidopsis* connected to an infrared gas analyser system (CIRAS 2, PP Systems, Amesbury, MA, USA) in experiment 3. Carbon fluxes were determined at steady state (approximately 15 min after light was switched on) under control temperature (20 °C) and HT (30 °C) but only in WW conditions, and under ambient  $\text{CO}_2$  (390 ppm) and light intensity (175  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PFD). Photosynthesis was measured on two to 15 plants at bolting on An-1, Col-0, Cvi-0, Ler, Mt-0 and Sha.

### Leaf ABA content

Leaf ABA ( $\text{ng g}^{-1}$  FW) was determined by radioimmunoassay (Quarrie *et al.* 1988) as previously described (Barrieu &

Simonneau 2000). Leaf samples were ground finely under liquid nitrogen, placed in distilled water (5 mL *per* mg FW) and immediately warmed at 70 °C for 5 min before shaking at 4 °C overnight. Extracts were then centrifuged at 16 000 *g* for 10 min at 4 °C; the supernatant was conserved at -20 °C and used for radioimmunoassay.

### Meteorological data at the geographical origin of the accessions

Meteorological data (temperature, precipitation, relative humidity, diurnal temperature range) at the geographical origin of the accessions were extracted from high-resolution gridded datasets of climate data (New *et al.* 2002). Mean monthly parameters were calculated for the main period of vegetative growth of *A. thaliana* from September to May (Hoffmann 2002).

### Data analysis

Statistical significance of trait variation was tested by three-way multivariate and univariate analyses of variance (MANOVA and ANOVA) with genotype, soil water content and air temperature as fixed factors. Post hoc comparison between treatments was performed with Kruskal–Wallis non-parametric test. Principal component analyses (PCAs) were performed to study the relationships between the traits and the effects of the temperature and soil water treatments. PCAs were performed on data from the experiment where higher number of both traits and genotypes were studied (experiment 2) and on standardized mean trait values by genotype and treatment ( $n = 36$ ) because traits were measured in very different units. Between- and within-treatment PCAs were performed on mean trait values to test for differences between treatments and focus on genotypic effects, respectively (Chessel, Dufour & Thioulouse 2004). The null hypothesis that there is no difference between treatments was tested with a randomization test (*randtest.between* in the *R/ade4* package). The procedure checks that the observed value of the between/total inertia ratio is higher than expected under the null hypothesis. The distribution of the between/total inertia ratio is obtained by permuting the rows of the data frame, that is means per genotype and treatment ( $n = 999$ ) and thus changing assignment to treatment group. Response ratios (R) between treated (T) and control (C) groups were calculated as  $R_{TC} = \text{mean trait value}_T / \text{mean trait value}_C$  to quantify the effects of the treatments for each genotype. Five values of response ratios were calculated to obtain the response to WD according to the control conditions (WD-20 °C/WW-20 °C), the response to WD at HT (WD-30 °C/WW-30 °C), the response to HT in WW conditions (WW-30 °C/WW-20 °C), the response to HT in WD conditions (WD-30 °C/WD-20 °C) and the response to the combination of HT and WD compared to the control conditions (WD-30 °C/WW-20 °C). The response ratio quantifies the proportionate change that results from an experimental

manipulation (Hedges, Gurevitch & Curtis 1999). Response ratios were log-transformed in the statistical analyses. We tested the significance of the relationships between traits, response ratios, coordinates of the genotypes of the PCA axes and climatic descriptors with correlation coefficients. All statistical tests were performed using R v.2.10 (R Development Core Team 2009).

## RESULTS

### Analysis of multiple plant traits reveals significant genotype by environment effects but predominant additive effects of HT and WD

ANOVAs explained from 25 to 85% of the total variance of 16 functional traits related to plant growth, structure and physiology, and the MANOVA explained 58% of the total variance in the multivariate dataset (Table 2). Across traits, there was a highly significant genotypic variability among accessions (18% of variance explained in the MANOVA; from 4 to 47% of variance explained across traits). Additionally, strong genotype by environment (soil water content, temperature or both) interactions were detected for all traits as indicated by highly significant first- and second-order interaction terms, highlighting the large natural phenotypic variability in the responses to both isolated and combined HT and WD. While significant for most of the traits, the effect of WD was not significant at the multivariate level. Interestingly, lack of significant interaction between water regime and temperature at the multivariate level and for most of the traits was indicative of prevailing additive effects of WD and HT (Table 2).

A PCA was performed in order to explore the multivariate pattern of effects of both isolated and combined HT and WD on the studied traits. First, second and third principal components (PC) explained 45, 25 and 9% of the total variance, respectively (Fig. 1; see Supporting Information Table S1 for variable loadings). Size-related traits contributed most to PC1 which opposed large plants with numerous vegetative leaves and high rate of leaf production to plants that had high reproductive mass allocation and thinner, more erect leaves with high SLA (Fig. 1a). Biomass allocation to the roots, epidermal cell density and stomatal density closely and negatively correlated with PC2. LDMC contributed less to this axis but contributed to most of the variation on third axis.

Projection of the accessions (Fig. 1b) showed that the four temperature-by-soil water treatments were significantly discriminated in the first factorial plane ( $P < 0.001$ ; permutation tests of between-treatments PCA), although the high genotypic variability was distinguishable as indicated by the distance of the accessions from the centroid of each treatment. Along PC1, plants grown under control conditions (20 °C air temperature; 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) were opposed to plants grown under combined HT and WD conditions (30 °C; 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil). As indicated by the position of the centroid of each treatment along PC1, all treatments reduced plant performance compared to control

conditions, and the combined stress was more detrimental to plants than isolated HT or WD. Isolated HT and WD treatments were significantly separated along PC2, indicating opposite effects of these stresses on traits related to this axis. Specifically, WD led to an increased biomass allocation to roots, a decrease in SLA and higher epidermal cell and stomata densities, whereas HT had opposite effects.

### *The combination of HT and WD is more detrimental to plant development than isolated effects, but differences between genotypes exist*

As shown by the PCA, rosette development dynamics were significantly affected by HT, WD and their combination (Fig. 2; Table 2; Supporting Information Fig. S1). In control conditions, the average of maximum rate of leaf production ( $R_{max}$ , leaf  $d^{-1}$ ) was 0.95 among genotypes and varied significantly from 0.75 in An-1 to 1.08 in Cvi-0 and Mt-0 (Supporting Information Fig. S2). The three treatments significantly reduced  $R_{max}$  (Fig. 2a; Table 2). Although the sensitivity of phenology to treatments varied significantly among *Arabidopsis* accessions, WD was, on average, more detrimental for leaf production (23% mean decrease) than HT (16% mean decrease; but see Lc-0 and Sha in Supporting Information Fig. S2a). Combining HT and WD had greater effects (40% mean decrease among genotypes) on  $R_{max}$  than isolated treatments (Fig. 2a). The duration of vegetative leaf production, which is highly related to flowering time in *A. thaliana*, also varied widely among accessions from 21 to 63 d in An-1 and Lc-0, respectively (Supporting Information Fig. S2b). Duration of leaf production and flowering time increased or decreased depending on accession and treatment leading to a highly significant second-order interaction term in the ANOVA (Table 2). While not significant in all accessions, WD tended to increase the duration of leaf production either at control or HT (non-significant water regime by temperature interaction in ANOVA; Table 2; Fig. 2b). By contrast, increasing air temperature tended to shorten the life cycle either in WW or WD conditions. As a result of their effects on plant growth dynamics, HT and WD significantly reduced total plant mass in all accessions but Cvi-0 and Lc-0 (Fig. 3; Table 2). On average, HT and WD similarly reduced total dry mass by twofold. Combining HT and WD (HT × WD) reduced plant size more severely than isolated stresses from 55% in An-1 to 91% in Ct-1 (Fig. 3 and 85% mean decrease). In some genotypes, plant dry mass tended to be less affected by isolated or combined HT and WD (An-1, Lc-0), while in others, it was less reduced only under HT (Cvi-0) or WD (Est-1, *Ler*). This resulted in weak relationships between response ratios to HT and WD for total dry mass (Supporting Information Fig. S3). However, the response ratio of HT × WD to control conditions ( $R_{HT \times WD/C}$ ) for the total dry mass was close to the sum of the response ratios of WD and HT to control conditions ( $R_{WD/C} + R_{HT/C}$ ) suggesting nearly additive effects. This was true for all accessions except Cvi-0, Lc-0 and Mt-0. These accessions apart, clear additive effects were indicated by a significant relationship between

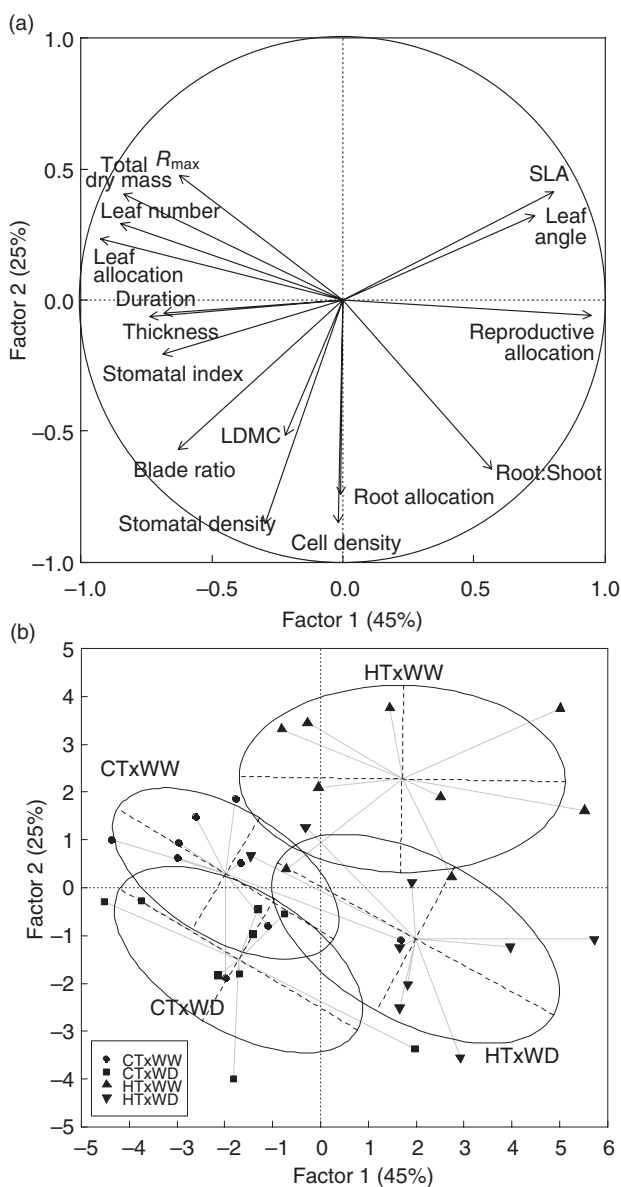
**Table 2.** Results of the partitioning of phenotypic variation among the natural genotypes grown in a full factorial design of contrasted soil relative water content (W, 0.35 and 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil in well-watered and water deficit treatment, respectively) and air temperature (T, 20 °C and 30 °C in control and high temperature treatment, respectively)

Trait	Genotype (G)	Soil water content (W)	Temperature (T)	W × T	G × W	G × T	G × W × T	R <sup>2</sup> (%)
d.f.	9	1	1	1	9	9	9	
MANOVA	18.1***	0.5	32.7***	0.5	6.7**	3.4	1.9	57.5
Plant growth and final size, and biomass allocation								
R <sub>max</sub> (leaf d <sup>-1</sup> ) <sup>a</sup>	19.5***	31.9***	14.2***	0.0	3.1**	5.2***	0.8	73.9
Growth duration (days) <sup>a</sup>	44.9***	2.6***	8.6***	0.0	2.1	8.9***	6.2***	73.3
Leaf number at flowering (leaf)	46.6***	1.2***	19.1***	0.1	2.1*	6.7***	3.0***	78.7
Total dry mass (mg)	33.4***	18.3***	19.3***	0.0	3.1***	6.3***	3.4***	83.8
Reproductive allocation	31.5***	0.01	16.1***	0.1	2.3	5.3**	4.6*	59.8
Root allocation	10.2***	9.1***	0.5	1.2	6.3*	4.2	4.2	25.6
Leaf allocation	35.6***	1.0**	21.8***	0.3	2.2	7.4***	2.8*	70.8
Root to shoot ratio	19.2***	6.0***	1.1*	0.4	7.3**	4.0	3.8	33.6
Leaf structure, anatomy and physiology								
Leaf insertion angle (°) <sup>a</sup>	4.2***	0.3	69.4***	0.1	0.6	1.8	1.1	76.6
Leaf blade ratio (%) <sup>a</sup>	18.9***	10.5***	29.5***	2.1*	2.1	3.1*	1.5	64.1
Specific leaf area (cm <sup>2</sup> g <sup>-1</sup> )	27.5***	4.0***	18.1***	5.3***	4.7**	3.2*	4.8**	67.6
Leaf dry matter content (mg g <sup>-1</sup> )	14.4***	13.9***	1.7**	6.5***	7.6***	2.3	6.0*	52.4
Leaf thickness (μm)	45.8***	0.9*	10.7***	0.1	1.0	9.0***	2.3	66.4
Cell density (cell mm <sup>-2</sup> )	46.5***	15.2***	6.8***	0.2	6.6***	6.9***	1.3	82.0
Stomatal density (st. mm <sup>-2</sup> )	29.9***	14.9***	24.8***	0.0	4.1***	3.0**	0.9	76.7
Stomatal index (% st. cell <sup>-1</sup> )	7.4**	1.1*	29.3***	0.1	3.6*	11.2***	3.4	52.6
ABA content (ng g <sup>-1</sup> FW)	9.8	20.2***	8.4***	0.7	17.3*	6.1	14.6*	60.5
Net photosynthetic rate (μmol CO <sub>2</sub> s <sup>-1</sup> m <sup>-2</sup> )	23.2**	–	23.3***	–	–	1.5	–	48.0

The percentage of variance explained ( $SS_x/SS_{total}$ ) and the level of significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ) for each factor and the interactions are indicated. Values are from the full model including all interactions. All non-significant terms are reported, but were removed from the final model. Hypothesis testing was based on Pillai-Bartlett statistic in the multivariate analysis of variance (MANOVA) and on  $F$ -ratios from type III mean squares for all ANOVAs.  $R^2$  is the proportion of total variance absorbed by the final model. All traits but  $R_{max}$  and leaf blade ratio were ln-transformed to fulfil ANOVA requirements. Net photosynthetic rate was not measured in WD conditions.

<sup>a</sup>Data are not available for Bay-0 and traits are not included in the MANOVA.

FW, fresh weight.



**Figure 1.** Principal component analysis on traits measured on nine *Arabidopsis* accessions grown under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. HT and WD treatments were applied after emergence of the first two true leaves and plants were harvested at first visible pod. Only the first two axes are shown. (a) Representation of the variables; LDMC, leaf dry matter content; SLA, specific leaf area. (b) Representation of the accessions with centres of gravity and lines connected to each accession shown for each condition. CT × WW (circles), CT × WD (squares), HT × WW (triangles) and HT × WD (upside-down triangles). Ellipses represent inertia ellipses of each treatment. Each inertia ellipse is centred on the means, its width and height are given by 1.5 times the standard deviation of the coordinates on axes, and the covariance sets the slope of the main axis (Thioulouse *et al.* 1997).

$R_{HT \times WDIC}$  and  $R_{WDIC} + R_{HTC}$  ( $r = 0.82$ ;  $P < 0.05$ ) with a slope not significantly different from one. Compared to other accessions, the growth of Mt-0 was less affected by the combination of HT × WD than by WD only (Fig. 3). To further investigate the genetic variability of responses to HT and WD, we analysed the ranking of the genotypes from the PCA performed on trait values. The rankings were well conserved on PC1 and PC2. The Spearman's coefficients of rank correlation varied from 0.58 to 0.92 (Supporting Information Table S2). This indicated that accessions which exhibited higher value of a trait compared to other accessions in control conditions conserved this advantage when stressed.

### *Biomass allocation to roots increases under WD and reproductive allocation increases at HT*

Biomass allocation also changed at the whole plant and leaf levels in response to isolated and combined WD and HT (Table 2; Fig. 3). Interestingly, at the whole-plant level, WD and HT had different effects on allocation to roots and to reproductive structures. WD resulted in a significant increase in biomass allocation to roots, but reproductive allocation did not change significantly (Fig. 4a). The reverse was found under HT where no changes were detected in the biomass allocation to roots, whereas a significant positive effect was observed on reproductive allocation.

### *WD and HT have different effects on leaf structure*

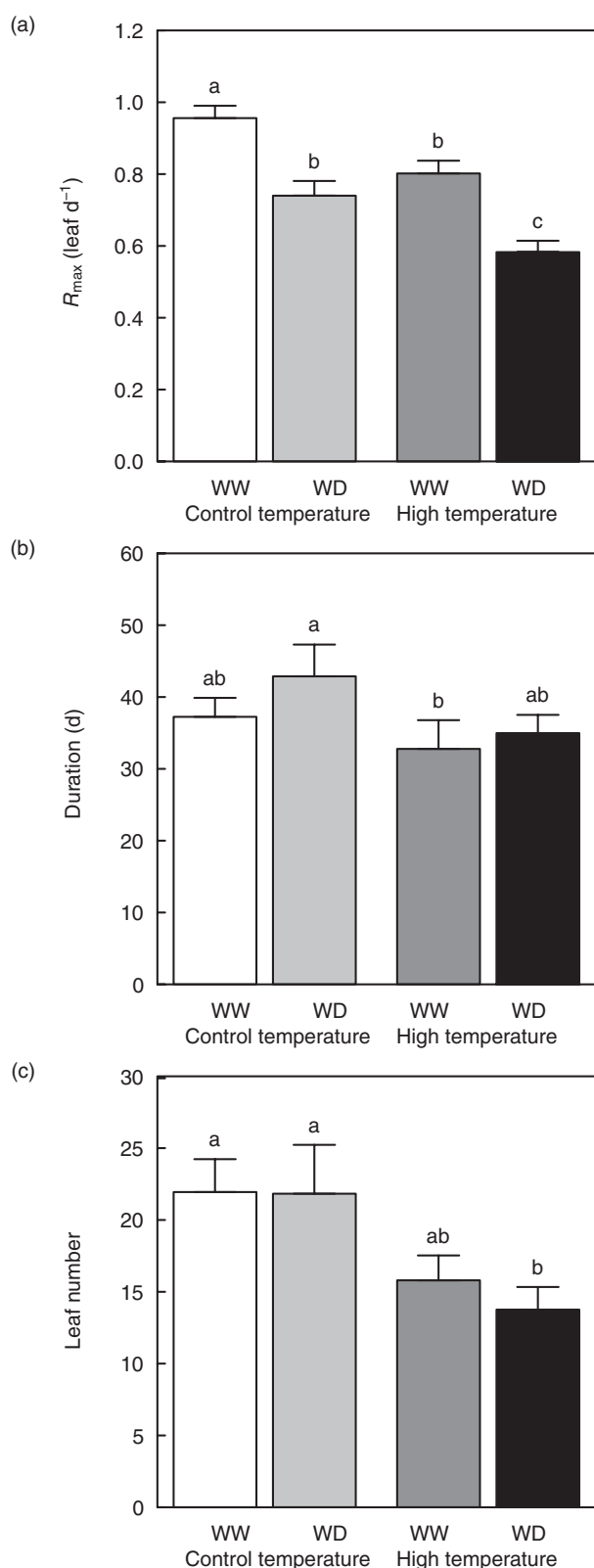
Leaves produced at HT tended to be thinner and had a higher SLA, while in WD, LDMC was increased (Fig. 4b–d; Supporting Information Fig. S2g–i). More precisely, SLA was much affected by HT in WW conditions and was significantly higher in all genotypes with little variation observed in WD, while LDMC tended to increase in response to WD, particularly at HT, and decrease under HT in WW conditions.

### *HT but not WD induces leaf hyponasty*

In all accessions, HT induced a highly significant increase in leaf insertion angle, that is hyponasty, associated with a significant reduction in the proportion of blade compared to petiole length (Fig. 4e,f; Table 2). WD had no significant effect on hyponasty either at control or HT. By contrast, a significant increase in blade ratio was found in response to WD, resulting in significant water by temperature interaction in the ANOVA for this trait (Table 2).

### *WD and HT have opposite but additive effects on leaf epidermis anatomy*

WD and HT had opposite effects on the cellular anatomy of leaf epidermis, but there was no water by temperature interaction as shown in the ANOVA (Table 2) indicating that the effects were globally additive. Across genotypes, cell and



**Figure 2.** Dynamics of leaf production under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Maximum rate of leaf production ( $R_{max}$ ) (a), duration of leaf production (b) and total leaf number (c). Bars are means + SE of nine accessions. Different letters indicate significant differences following Kruskal–Wallis test ( $P < 0.05$ ).

were detected (Table 2; Supporting Information Fig. S21–n). HT resulted in lower stomatal index (Fig. 5c). On the contrary, stomatal index tended to increase in response to WD, but the effect of this treatment was not detectable in several genotypes.

### *Photosynthesis is reduced at HT and ABA content increases under WD and HT*

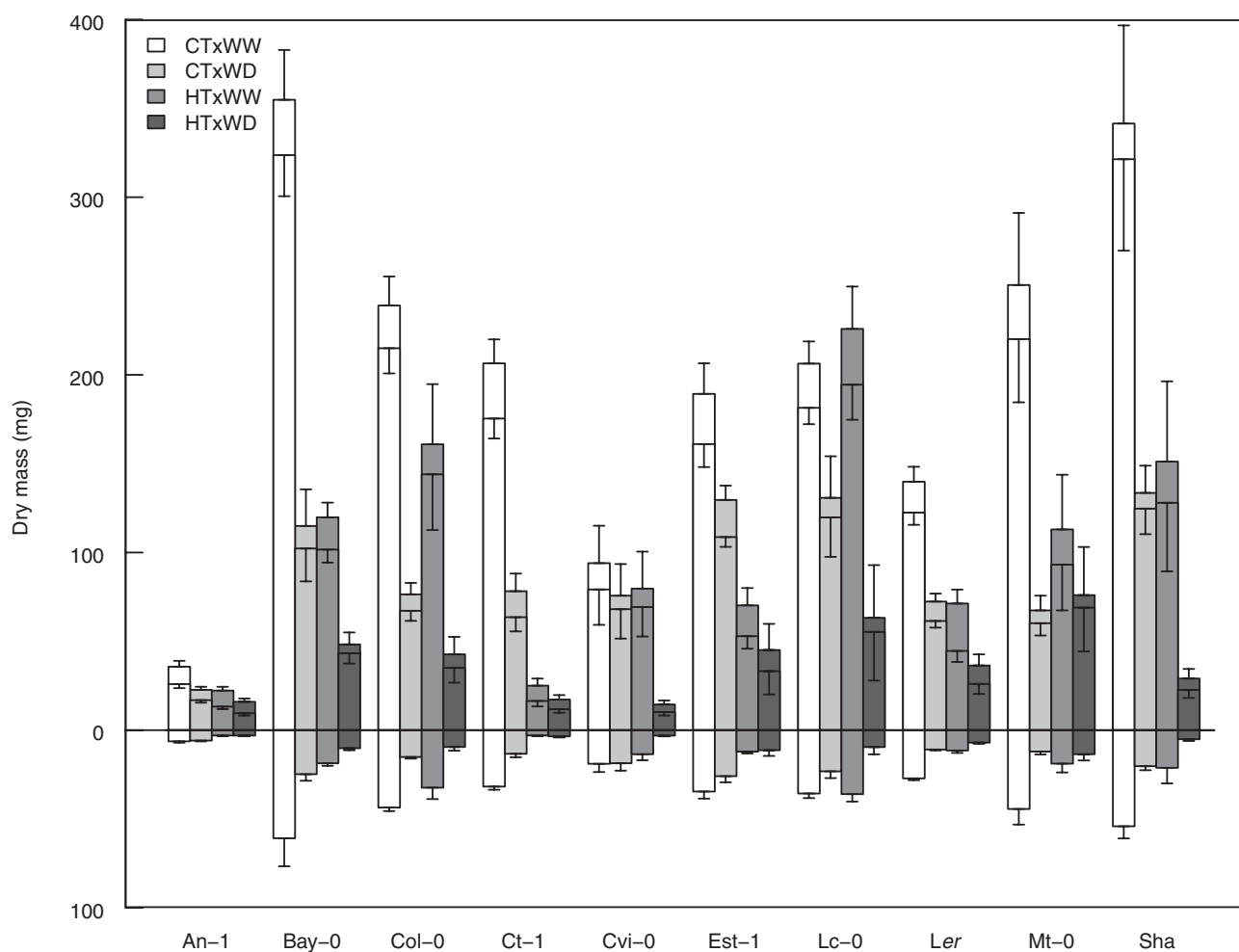
In WW conditions, net photosynthetic rate was significantly reduced by HT from  $3.95 \pm 0.73$  at 20 °C to  $3.30 \pm 0.56 \mu\text{mol CO}_2 \text{ s}^{-1} \text{ m}^{-2}$  at 30 °C (Fig. 6a; Table 2). No significant genotype by temperature interaction was detected ( $P = 0.29$ ; Table 2). Across all genotypes, leaf ABA content was significantly increased under WD and HT, and it was even more increased in response to the combination of the two stresses WD and HT (Fig. 6b).

### **Do responses to HT and WD relate to accessions climatic origin?**

Beyond mean responses to single or combined treatments, the accessions studied herein displayed a range of sensitivities for their different traits. We explored whether any part of the responses of the accessions was related to the climatic conditions at geographical origin of the populations in which they were collected. The data from the PCA were used in order to reduce the number of comparisons and therefore the risk of type I error.

For each treatment, no trend was observed between accessions coordinates on PC1 from the PCA on trait values and mean monthly temperature at geographical origin of the populations. However, for plants grown under HT in WW conditions, a positive trend was found between coordinates on PC2 and temperature of origin (Fig. 7a). Inspection of Fig. 7 revealed that the accession from Cape Verde Island (Cvi-0) had a contrasted response compared to the other accessions. When excluding Cvi-0 from the analysis, the correlation was high and significant ( $r = 0.80$ ;  $P < 0.01$ ; Fig. 7a). The collection site of this accession presents the higher temperature, although it was reported that Cvi-0 has been collected at 1200 m asl (Tonsor *et al.* 2008), thus possibly encountering lower temperatures. As seen earlier, PC2 was negatively correlated to stomatal and cell density and biomass allocation to roots. Therefore, the accessions that originate from sites with higher temperature tend to have less stomata per unit leaf surface, and to allocate less biomass to the roots than accessions from colder sites when cultivated under HT (Fig. 7b,c).

stomata densities increased in response to WD both at control temperature and HT, whereas these traits tended to decrease in response to HT (Fig. 5). Stomatal index exhibited much less variation, but genotype and treatment effects



**Figure 3.** Plant dry mass under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Bars are means  $\pm$  SE ( $n = 4$  to 9) for the roots (below), vegetative leaves (intermediate) and reproductive stems (top) of 10 *Arabidopsis* accessions.

Positive trends were also found between the coordinates on PC2 from the PCA on trait values and mean monthly precipitation from September to May in all treatments ( $r = 0.40$  to  $0.73$ ). While not statistically significant, this corresponded to a stronger reduction in stomatal density under WD, HT or both for accessions originating from sites with high precipitations ( $r = -0.36, -0.51$  and  $-0.56$ , respectively).

### Relationships between plant traits and tolerance to HT and WD

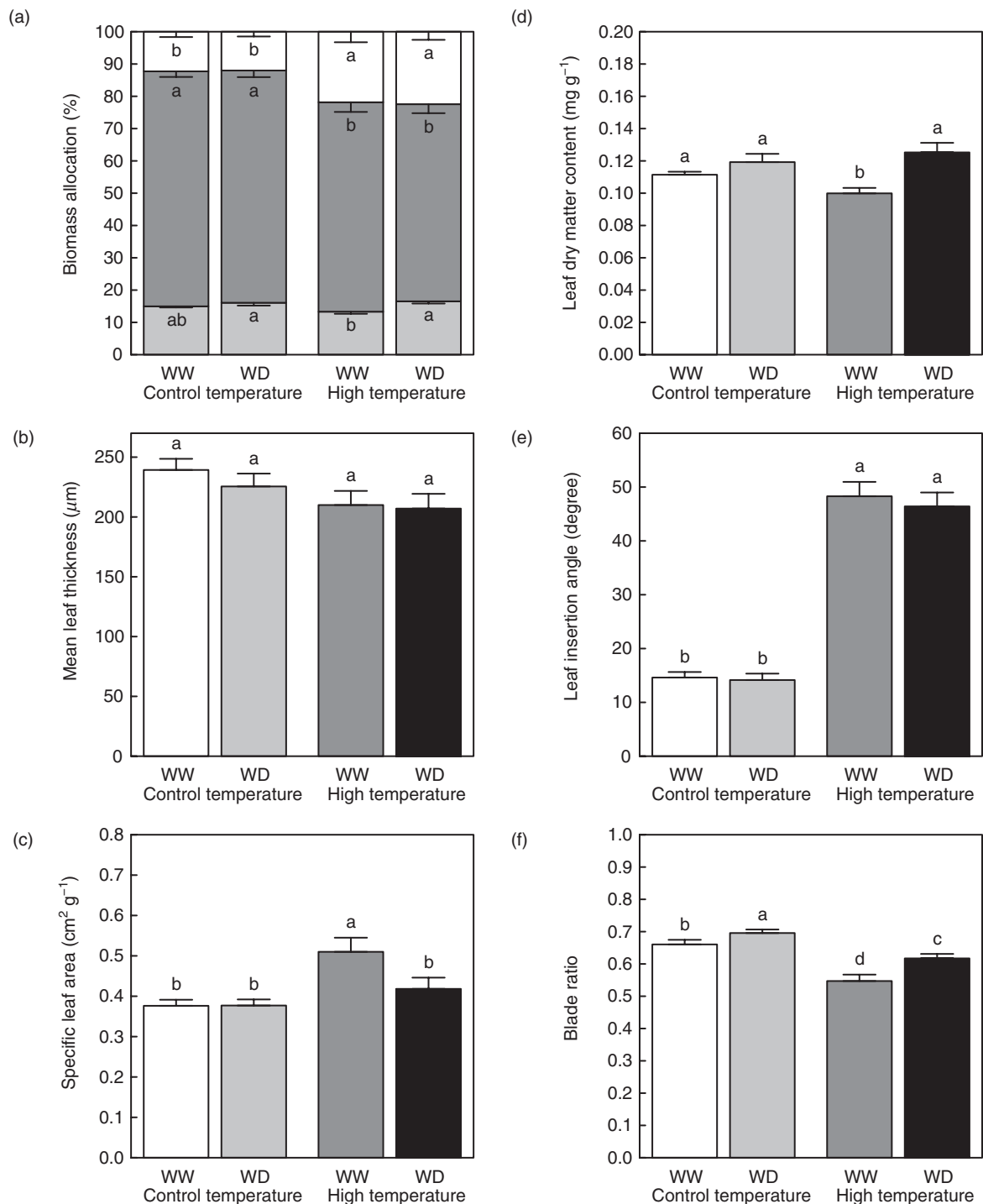
We explored the relationships between plant traits as measured in controlled conditions and accessions response to HT and WD. A negative correlation was found between absolute plant size in controlled conditions and the response ratio of plant size to the treatments. This trend was significant in response to WD ( $r = -0.73$ ;  $P = 0.03$ ; Fig. 8a) but not to HT ( $r = -0.27$ ;  $P = 0.48$ ) or the combination of HT and WD ( $r = -0.50$ ;  $P = 0.17$ ). Thus, stunted accessions (e.g. An-1) tend to be more tolerant to WD. Furthermore,

the root-to-shoot ratio in controlled conditions was positively correlated with the response ratio of plant size to WD ( $r = 0.68$ ;  $P = 0.04$ ; Fig. 8b) and with the response ratio of leaf production rate under combined HT  $\times$  WD ( $r = 0.72$ ;  $P = 0.04$ ). Thus, accessions with bigger root compartment relative to shoot tended to better maintain growth under WD, and to keep producing leaves at the same rate as control under combined stresses.

## DISCUSSION

### WD and HT: independent or interacting responses?

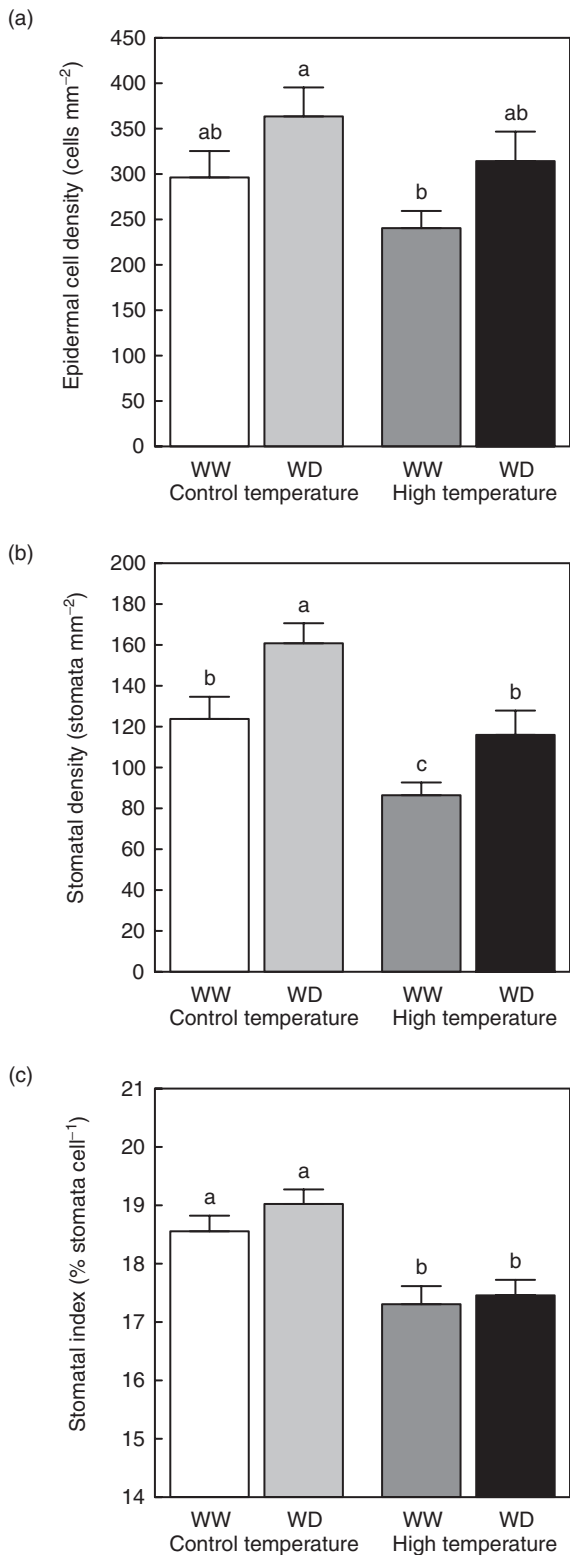
Complex interactive responses can occur in plants experiencing multiple environmental stresses (Mittler 2006). Here, we report the single or combined effects of soil WD and HT on a large set of plant traits from the cellular to the whole-plant levels in a collection of accessions of the model plant *A. thaliana*. Plant growth was significantly reduced under HT and WD, and their combination was more



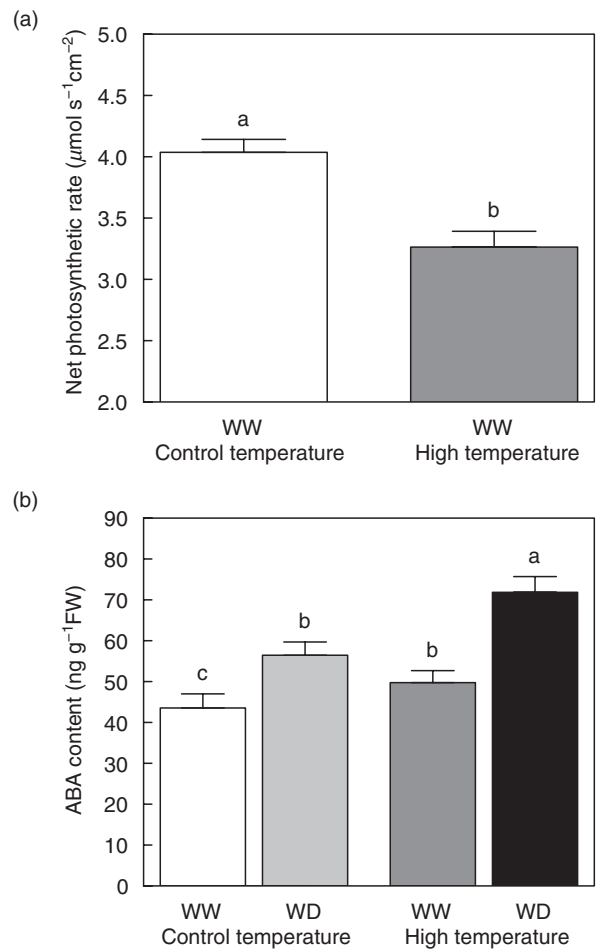
**Figure 4.** Biomass allocation and leaf morphology under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Dry mass allocation to the roots (below), vegetative leaves (intermediate) and reproductive stems (top) (a), leaf thickness (b), specific leaf area (c), leaf dry matter content (d), leaf insertion angle (e) and blade ratio (f). Bars are means ± SE of nine accessions. Different letters indicate significant differences following Kruskal–Wallis test ( $P < 0.05$ ).

detrimental to plant performance as also described in previous studies (Xu & Zhou 2006; Prasad *et al.* 2008). Interestingly, single trait as well as multiple traits analyses revealed that the combined effects of these two stresses

were globally additive. This held true for traits responding in the same (e.g. plant mass) or reverse (e.g. stomatal density) directions to the two stresses and suggests a certain degree of independency between the mechanisms involved



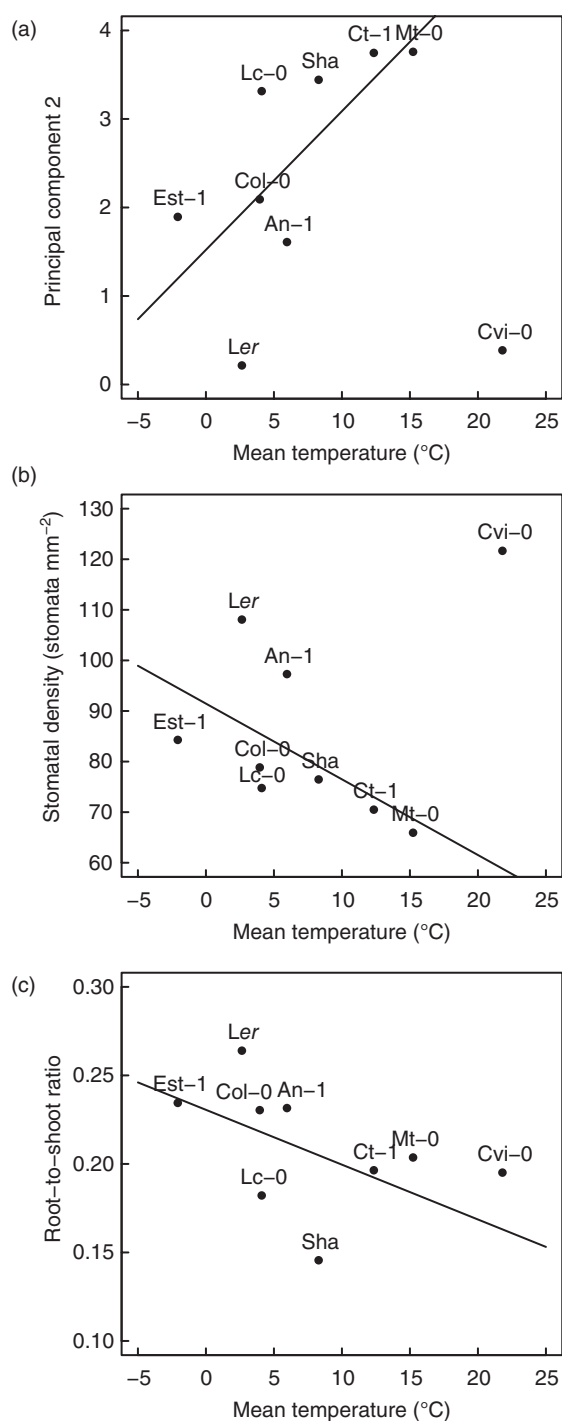
**Figure 5.** Leaf epidermal anatomy under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Cell density (a), stomatal density (b) and stomatal index (c). Bars are means + SE of nine accessions. Different letters indicate significant differences following Kruskal–Wallis test ( $P < 0.05$ ).



**Figure 6.** Net photosynthetic rate (a) and abscisic acid (ABA) content (b) under control (CT, 20/17 °C day/night) and high temperature (HT, 30/25 °C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Bars represent means + SE of nine accessions. Different letters indicate significant differences following Kruskal–Wallis test ( $P < 0.05$ ). Net photosynthetic rate was not measured in WD conditions. FW, fresh weight.

in the responses to WD and HT applied herein. Some traits were specific of the response to either WD or HT. This was the case for biomass allocation to roots which increased in response to WD, and conversely for reproductive allocation, leaf insertion angle and SLA which significantly increased in response to HT (Xu & Zhou 2006). However, among the large number of traits investigated, no single trait was affected only by the combination of HT and WD. The impact of the combined stresses has been rarely studied. In wheat and sorghum, Machado & Paulsen (2001) found that plant water status in response to HT was highly dependent on soil water availability. The work by Rizhsky and collaborators showed that some molecular responses were specific to the combination of heat and drought compared to either stress alone (Rizhsky *et al.* 2002, 2004). Yet our study is, to our knowledge, the first addressing this issue in different ecotypes and using a broad range of growth, developmental and physiological traits, and the lack of



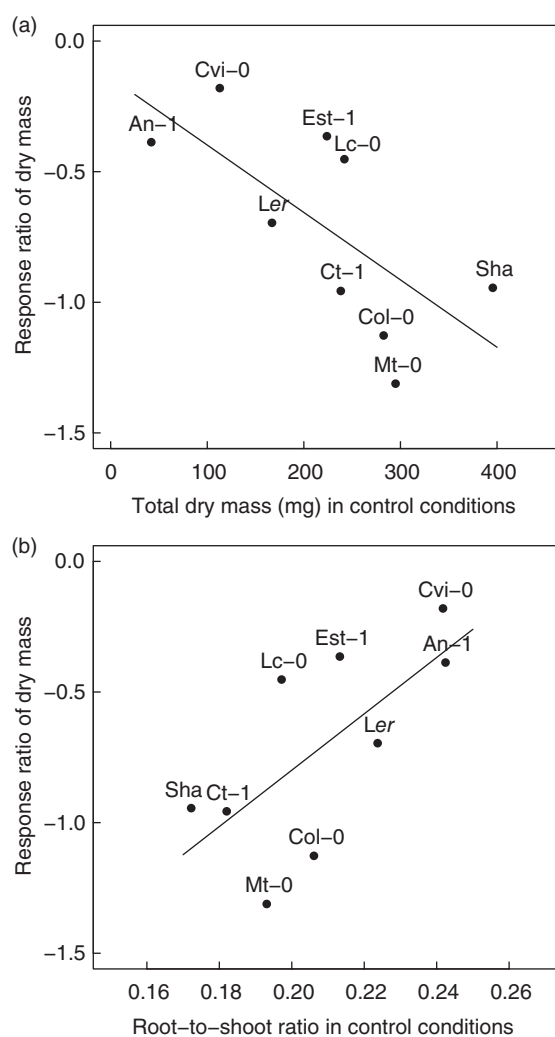


**Figure 7.** Relationships between mean temperature at the collection sites of nine *Arabidopsis* accessions and (a) PC2 coordinates (Fig. 1), (b) stomatal density and (c) root-to-shoot ratio under high temperature (30 °C) but well-watered (0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions.

HT × WD interaction is the rule for most of them, at least for the moderate levels of stresses applied during the whole plant cycle.

As generally found, plant growth dynamics (leaf production and leaf expansion) were significantly impaired in

response to HT (Loveys *et al.* 2002) and WD (Granier *et al.* 2006; Hummel *et al.* 2010), leading to reduced plant size at reproductive stage and therefore reduced seed production (Aarssen & Clauss 1992). However, the two stresses had contrasting effects onto the timing of reproduction. As commonly found in natural and crop species (McMaster *et al.* 2009), WD delayed reproduction, but contrasted effects on final leaf number were found across accessions. By contrast, under HT, fewer leaves were produced when early reproduction occurred. Early reproduction following a moderate increase in temperature has been previously reported in *A. thaliana* (Balasubramanian *et al.* 2006) and other species (Barnabas, Jäger & Fehér 2008). However, very sparse data are available on the combined effects of HT and WD on reproductive phenology in natural species (but see Barnabas *et al.* 2008 for a review in cereals). Here, we found that the effects were globally additive in such a way that WD also delayed flowering under HT.



**Figure 8.** Relationships between the response ratio of total dry mass to water deficit (0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil/20 °C soil water content/air temperature) of nine *Arabidopsis* accessions and (a) the total dry mass and (b) root-to-shoot ratio in control conditions (0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil/20 °C).

Although the majority of plants reached the flowering stage and a significant increase in biomass allocation to the reproductive stem was found under HT, flower abortions were clearly visible on later reproductive stages and very few pods reached maturity (not shown). The fecundity of the plants was particularly impaired under combined stresses. This was not surprising since reproductive structures are particularly sensitive to heat stress (Zinn, Tunc-Ozdemir & Harper 2010) and even more to combinations of heat and drought (Barnabas *et al.* 2008). Notably, HTs (31–33 °C) very close to that experienced here (30 °C) have been shown to be sufficient to impair anthers development in non-acclimated plants of *A. thaliana* (Sakata *et al.* 2010). Apparently, vegetative acclimation to long-lasting treatments as experienced here did not change this response.

### Is genetic variability of responses related to the climate of origin?

In our study, except the young seedling stage (before the emergence of the first true leaves), plants developed entirely under HT, WD or both. This may have led to acclimation processes possibly reinforcing plant tolerance to these stresses. Applying steady-state contrasted temperatures would also have produced different responses than those identified in the case of acute increase of temperature applied at a particular developmental stage as it is largely found in the literature. Nevertheless, a high genotypic variability in traits values was observed in the different growing conditions, and a significant genotype by environment interaction was found. This is not surprising given that the chosen accessions originated from a wide range of environments with varying temperature and drought constraints. A high variability of traits related to growth and phenology has been identified in natural populations of *A. thaliana* (Montesinos-Navarro *et al.* 2011). And genotypic variability among natural accessions has previously been identified for traits related to adaptation to WD (McKay, Richards & Mitchell-Olds 2003) and temperature (Tonsor *et al.* 2008). Here, we applied a HT level within the physiological range of *A. thaliana* and close to the basal thermotolerance of the accession Col-0 (Ludwig-Muller *et al.* 2000). Unfortunately, as far as we know, basal thermotolerance has not been consistently evaluated for other accessions than Col-0. Therefore, we cannot exclude that the variability of responses to HT observed here between the accessions was related to contrasted basal thermotolerance, which could also depend on the environment encountered in their habitat of origin. Few relationships between plant tolerance to HT and the climatic environment at the collection site of the accessions were found in this study. This is in accordance with Loveys *et al.* (2002) who found no relationship between thermal origin of the accessions and the production of dry matter in response to increasing temperature at the interspecific level. However, a lack of association could arise from the small number of accessions considered in our study. In a more geographically restricted study but including a large set of *Arabidopsis* natural populations,

Montesinos-Navarro *et al.* (2011) showed that the variation of traits exhibited in controlled conditions was consistent with the temperature and water constraints encountered at the collection sites along an altitudinal gradient, pointing towards a likely adaptive differentiation of the populations to the environmental conditions. Here, we found that accessions that originate from sites with higher mean temperature during the vegetative growth tend to have less stomata per unit leaf surface, and to allocate less biomass to the roots than accessions from colder sites when grown under HT.

### Stomatal density and plant response to HT and WD

Despite the prevailing opinion that stomatal density would increase in response to HT (Wahid *et al.* 2007), data from the literature are not unanimous (see Luomala *et al.* 2005). Indeed, it is most likely that stomatal density depends on tight interactions between plant water balance (water status and transpiration) and the environmental conditions, particularly relative humidity and VPD ( $VPD_{air}$ ) encountered by the plant during leaf growth (Lake & Woodward 2008). Assuming that conditions favouring expansion dilute stomata at the leaf surface, increases in humidity in the vicinity of the plant are expected to reduce stomatal density. In this study, the possible effects of  $VPD_{air}$  on stomatal density at HT were excluded since  $VPD_{air}$  was maintained equal between the control (20 °C) and the HT (30 °C) treatment. In order to fulfil this condition of constant  $VPD_{air}$ , air relative humidity was maintained higher under HT (85%) than under control (65%) conditions, possibly favouring the development of leaves with lower stomatal density at HT compared to control temperature. This was observed despite the significantly higher transpiration rate under HT compared to control temperature (Supporting Information Fig. S4). In addition, our results unequivocally show that soil WD led to increases in stomatal densities either at control or HT, thus counteracting the effects of HT. The same trend of decreasing and increasing stomatal density in response to HT and WD, respectively, was found in almost all genotypes. Despite the fact that  $VPD_{air}$  was maintained equal between the two temperature treatments, accelerated depletion of soil water or lower leaf water potential may have interfered with plant responses at HT due to higher rates of transpiration (Machado & Paulsen 2001; Supporting Information Fig. S4). Interestingly, relationships were found between stomatal density and meteorological conditions at the collection sites. Stomatal density was lower in accessions collected in warmer sites and/or sites with higher amount of precipitations, particularly when considering the responses to HT and WD.

Contrary to what was suggested by Lake & Woodward (2008), we found no relationship between ABA content in the rosette leaves and stomatal density. We cannot exclude a differential response of abaxial versus adaxial leaf epidermis in our experiments (see Luomala *et al.* 2005); however,

we observed that stomatal densities of both sides of the leaves are correlated either under WW or WD conditions (Vile & Pervert, unpublished results).

### Similarities between responses to HT and low light

It is noteworthy that some of the specific responses to HT were also characteristic of responses to low light intensity. For instance, it is well known that SLA increases and LT decreases in response to low light (Poorter *et al.* 2009), and that shade leaves have higher SLA and are thinner than leaves exposed to direct sun light (McMillen & McClendon 1983). Chabot & Chabot (1977) reported that decreasing light and moderately elevated temperature had similar effects on thickness. In *Arabidopsis*, a clear similarity between the responses to light and HT resides also in hyponastic growth, that is the increase in leaf insertion angle (Van Zanten *et al.* 2009). These authors reported very similar trends of variation in leaf angle in response to HT and low light, and we have recently shown that the hyponastic response to HT can be reversed by increasing light intensity (Vasseur, Pantin & Vile 2011). Taken together, these results suggest that part of the responses to a moderate heat stress could be associated to a defect in carbon acquisition through photosynthesis, which is impaired under HT, and/or an increased competition for carbon use due to enhanced physicochemical processes and increased protection mechanisms (notably heat shock proteins; Heckathorn *et al.* 1996). Accordingly, tolerance to warm temperatures is increased at high CO<sub>2</sub> concentration in C3 plants (Huxman *et al.* 1998; Taub, Seemann & Coleman 2000), and decreased at low nitrogen supply due to a limited production of nitrogen-costly heat shock proteins (Heckathorn *et al.* 1996). The interactive effects of HT and light on plant functioning were analysed here under lower light than encountered in natural conditions. To test whether our results would hold under higher light conditions as found in the nature, especially at HT, experiments should be performed at higher light intensities. Interactions between WD, HT and light also remain to be investigated (Vasseur *et al.* 2011).

### Inherent trait variation and plant tolerance to HT and WD

Ecological research has engaged major efforts to identify plant traits, as measured in controlled or natural conditions, that could be good predictors of plant responses to changes in their environment (Grime 2001; Vile, Shipley & Garnier 2006; Violle *et al.* 2007). Here, we found a trade-off between plant size in control conditions and tolerance to WD. A similar negative relationship between plant size and plant tolerance to WD was found in an analysis of 20 accessions capturing much of the genetic variation of *A. thaliana* worldwide (Clark *et al.* 2007) and a new collection of 88 accessions from Europe and Asia (Bouteillé *et al.*,

unpublished results;  $r = -0.54$  and  $-0.25$ ;  $P = 0.013$  and  $0.022$ , respectively). A re-analysis of the data from Bouchabke *et al.* (2008) also showed a significant negative relationship between total leaf area in WW conditions and its response to a mild WD applied for 10 d ( $r = -0.49$ ;  $P = 0.014$ ). Interestingly, we found a similar ranking of responses to WD for the six common accessions (but Sha to a lesser extent) between Bouchabke *et al.* (2008) and our study. Such a trade-off between plant size and the response ratio to WD was also found in a re-analysis of the data of a recent study on stress-related specific mutants of *Arabidopsis* (Skiryecz *et al.* 2011), although plant size variation between lines was weak ( $r = -0.43$ ;  $P = 0.014$ ). These authors report that growth reduction caused by stress was independent of plant size under control conditions, but they used the relative response of mutants compared to the wild type, not the response ratio for each line. A first explanation for this trade-off would reside in the fact that large plants consume more water and therefore experience greater water shortage. However, the experimental procedure used in the present study as well as in Bouchabke *et al.* (2008) and in Skiryecz *et al.* (2011), that is a daily irrigation to adjust the soil water content, is unlikely to have favoured small plants that consume less water. A trade-off between plant size and plant tolerance to WD is in accordance with the results of He *et al.* (2010) that populations of *Centaurea stoebe* with inherently bigger plant size are more susceptible to stressing (water and nutrient) conditions. In contrast to these authors, who did not observe any relationship with other traits than plant size, here, we found a positive relationship between the root-to-shoot ratio and plant tolerance to WD which could give a proportionate advantage under inherent water shortage.

On the other hand, the negative trend between plant size and *Arabidopsis* tolerance to HT was weaker and not significant. No single trait was identified as a good predictor of plant response to HT. Some elements suggest that changes in leaf inclination could participate to thermotolerance adjustments by reducing intercepted light and hence tissue temperature (Salvucci & Crafts-Brandner 2004). Although leaf insertion angle increased in response to HT and this response varied between accessions, in our data, hyponasty was not related to thermotolerance. Furthermore, in contrast to the results of Van Zanten *et al.* (2009), no relationship was observed between the change in leaf angle in response to HT and the diurnal temperature range at the geographical origin of the accessions. This discrepancy could in part be explained by the higher but shorter temperature treatment experienced in Van Zanten *et al.* (38 °C during 7 h) compared to our study (30 °C during c. 15 d).

Finally, plant tolerance to WD under HT, in terms of plant size reduction, was also related to plant size in WW and control temperature conditions albeit the relationship was weaker than for WD under control temperature. Thus, inherent plant size would participate in soil–water–plant relationships to a larger extent than to the response to increasing temperature.

## CONCLUSION

Despite the likely interactive processes involved in plant response to HT and WD, here, we showed that at least moderate levels of these two stresses have additive effects on a large set of plant traits related to growth and development in the model species *A. thaliana*. This would have important consequences for modelling plant growth under combined stresses. Some traits were affected only by one or the other stress, highlighting the specific sensitivity of some processes such as reproduction in response to HT and resources allocation for a better water acquisition in response to water deprivation. In natural environments, variation in temperature and water availability can act together or independently on co-varying traits and on the distribution of plant species. It was therefore not surprising to find a significant natural variation in *Arabidopsis* tolerance to HT and WD applied separately or in combination. Genetic variability in the responses of several traits to the different stresses accompanied this natural range of tolerances and was in good correspondence with some characteristics of the climatic origin of the natural populations. This opens several avenues to explore the underlying physiological processes shaping the distribution of this and other species.

## ACKNOWLEDGMENTS

We thank J.-J. Thioux, M. Dauzat, A. Bédicé and G. Rolland for the technical support during the experiments. We thank A. Skirydz for providing data, and two anonymous referees for improvements to the manuscript.

This work was supported by Agron-Omics, a European sixth framework integrated project (LSHG-CT-2006-037704). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received 28 July 2011; received in revised form 29 September 2011; accepted for publication 4 October 2011

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Leaf production dynamics in *A. thaliana* Col-0.

**Figure S2.** Mean trait values by genotypes and treatments.

**Figure S3.** Correlation matrix of response ratios for total dry mass.

**Figure S4.** Night and day transpiration rates of Col-0 and *Ler* accessions.

**Table S1.** Loadings of the variables included in the PCA on mean trait values.

**Table S2.** Correlations between genotypes coordinates on first and second principal components from the PCA performed on trait values.

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## Supporting Information

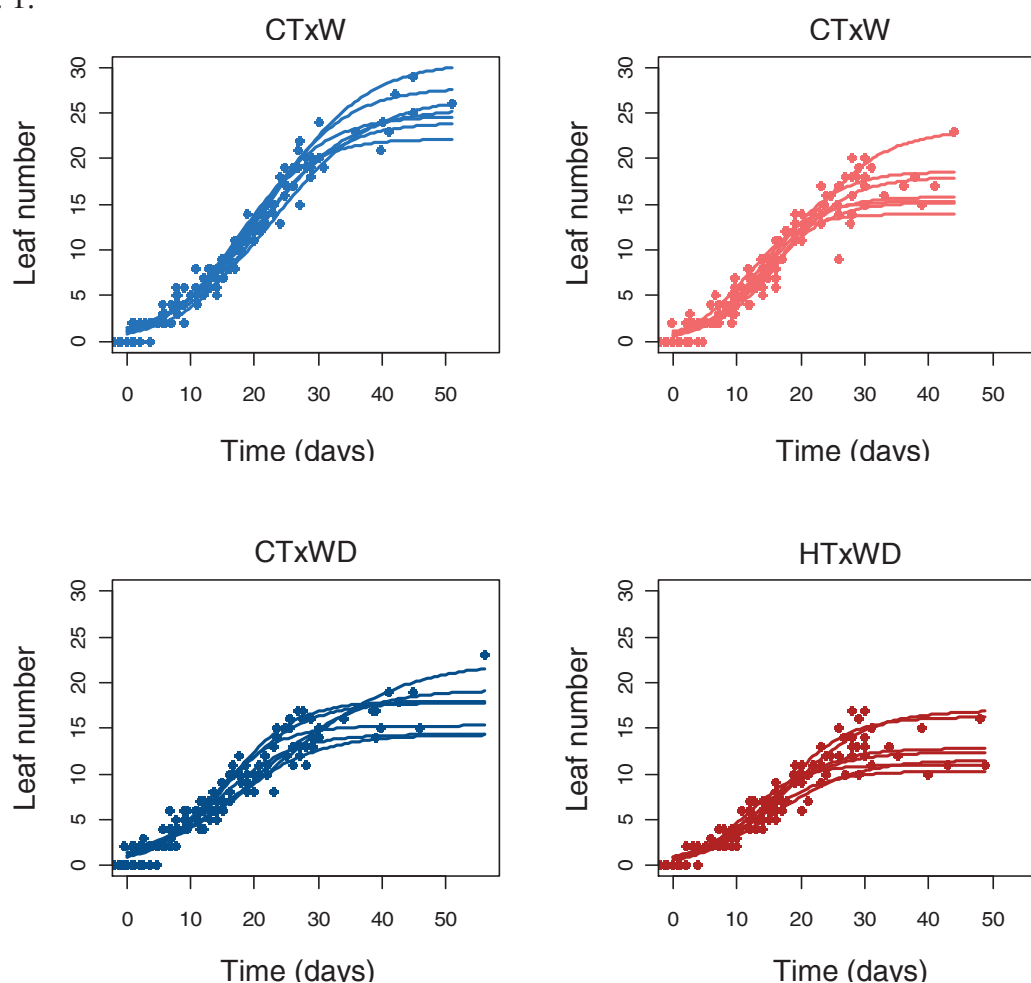
**Table S1.** Loadings of the variables included in the PCA on mean trait values per genotype and treatment. All variables have been log-transformed.

Trait	Factor 1	Factor 2	Factor 3
Leaf number at flowering (leaf)	-0.849	0.291	-0.249
Total dry mass (mg)	-0.836	0.405	0.017
Specific leaf area (cm <sup>-2</sup> g <sup>-1</sup> )	0.806	0.413	0.232
Leaf dry matter content (mg DM g <sup>-1</sup> FM)	-0.218	-0.516	-0.767
Leaf thickness (μm)	-0.684	-0.048	0.416
Reproductive allocation (%)	0.946	-0.058	-0.031
Root allocation (%)	-0.008	-0.743	-0.132
Leaf allocation (%)	-0.925	0.233	0.035
Root to shoot ratio	0.585	-0.661	0.017
Cell density (cells mm <sup>-2</sup> )	-0.019	-0.851	0.143
Stomatal density (st. mm <sup>-2</sup> )	-0.298	-0.854	0.204
Stomatal index (% st. cell <sup>-1</sup> )	-0.686	-0.208	0.156
Maximum leaf production rate (R <sub>max</sub> , leaf d <sup>-1</sup> )	-0.624	0.475	0.062
Duration of leaf production (d)	-0.737	-0.063	-0.482
Leaf insertion angle (°)	0.732	0.324	-0.283
Blade ratio	-0.628	-0.573	0.247

**Table S2.** Spearman's coefficients of rank correlation between genotypes coordinates on first (above diagonal) and second (below diagonal) principal components from the within-treatment PCA performed on trait values under control (CT, 20/17°C day/night) and high temperature (HT, 30/25°C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Coefficients in bold typeface were significant at \**P* < 0.05, \*\**P* < 0.01. *n* = 9.

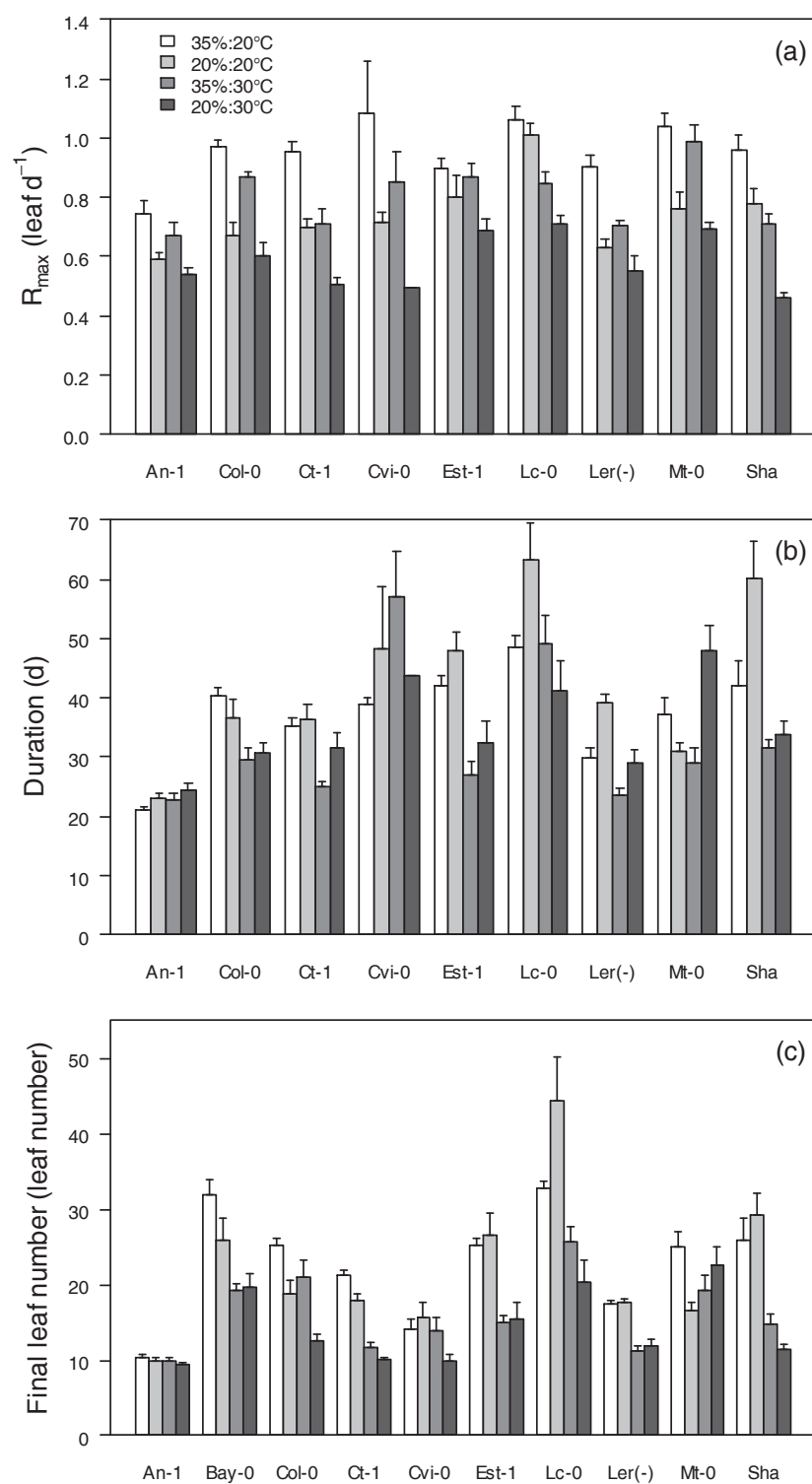
CTxWW	<b>0.73*</b>	<b>0.68*</b>	<b>0.80*</b>
<b>0.92**</b>	CTxWD	0.58	<b>0.65*</b>
<b>0.85**</b>	<b>0.73*</b>	HTxWW	0.58
<b>0.77*</b>	<b>0.63</b>	<b>0.73*</b>	HTxWD

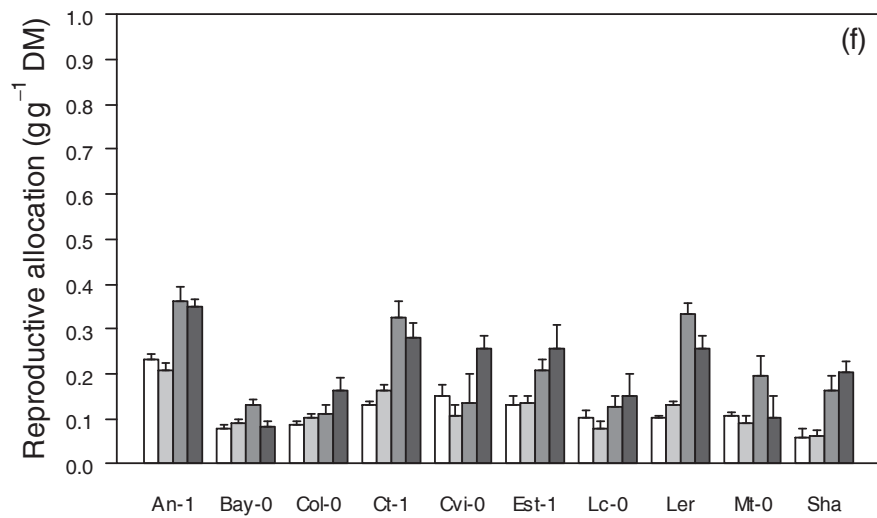
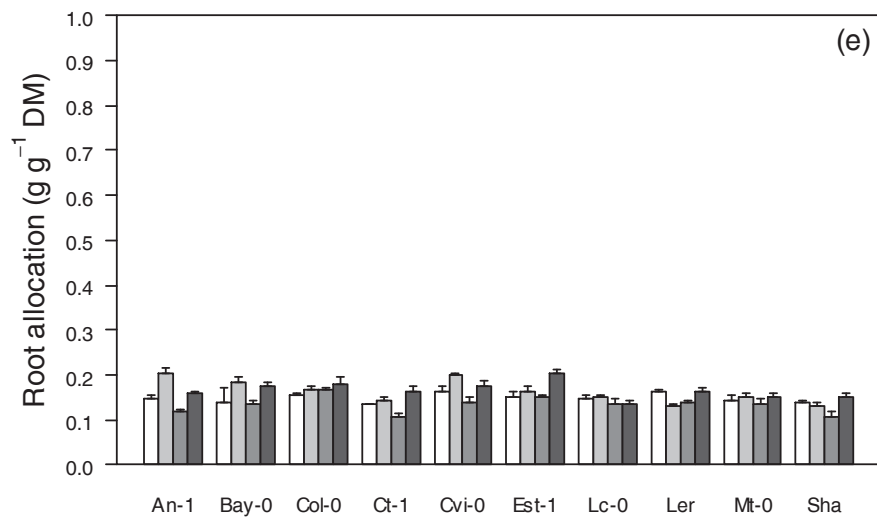
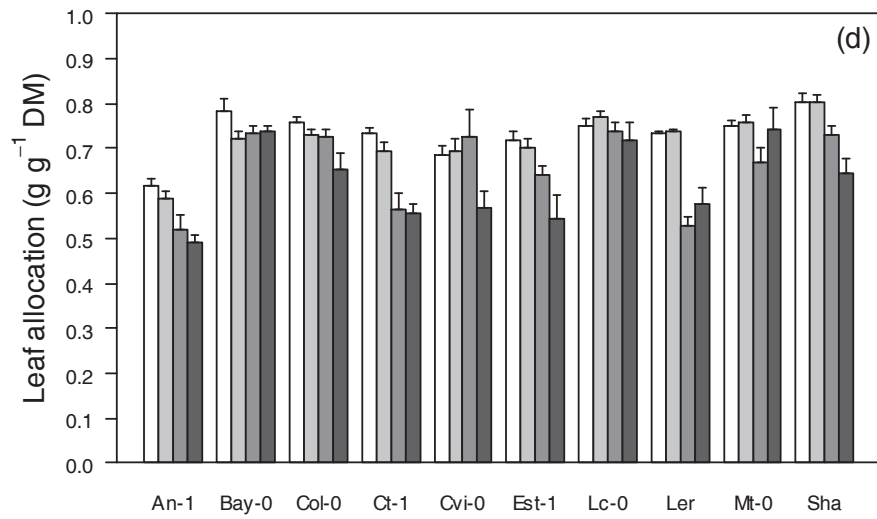
**Figure S1.** Production of leaves of *A. thaliana* Col-0 from cotyledonous stage to silique maturation. Each fitted curve represents one individual plant grown under control (CT, 20/17°C day/night) and high temperature (HT, 30/25°C day/night), and in well-watered (WW, 0.35 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 0.20 g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions. Curve fitting of leaf production over time (days from cotyledonous stage) was calculated according to Eq. 1.

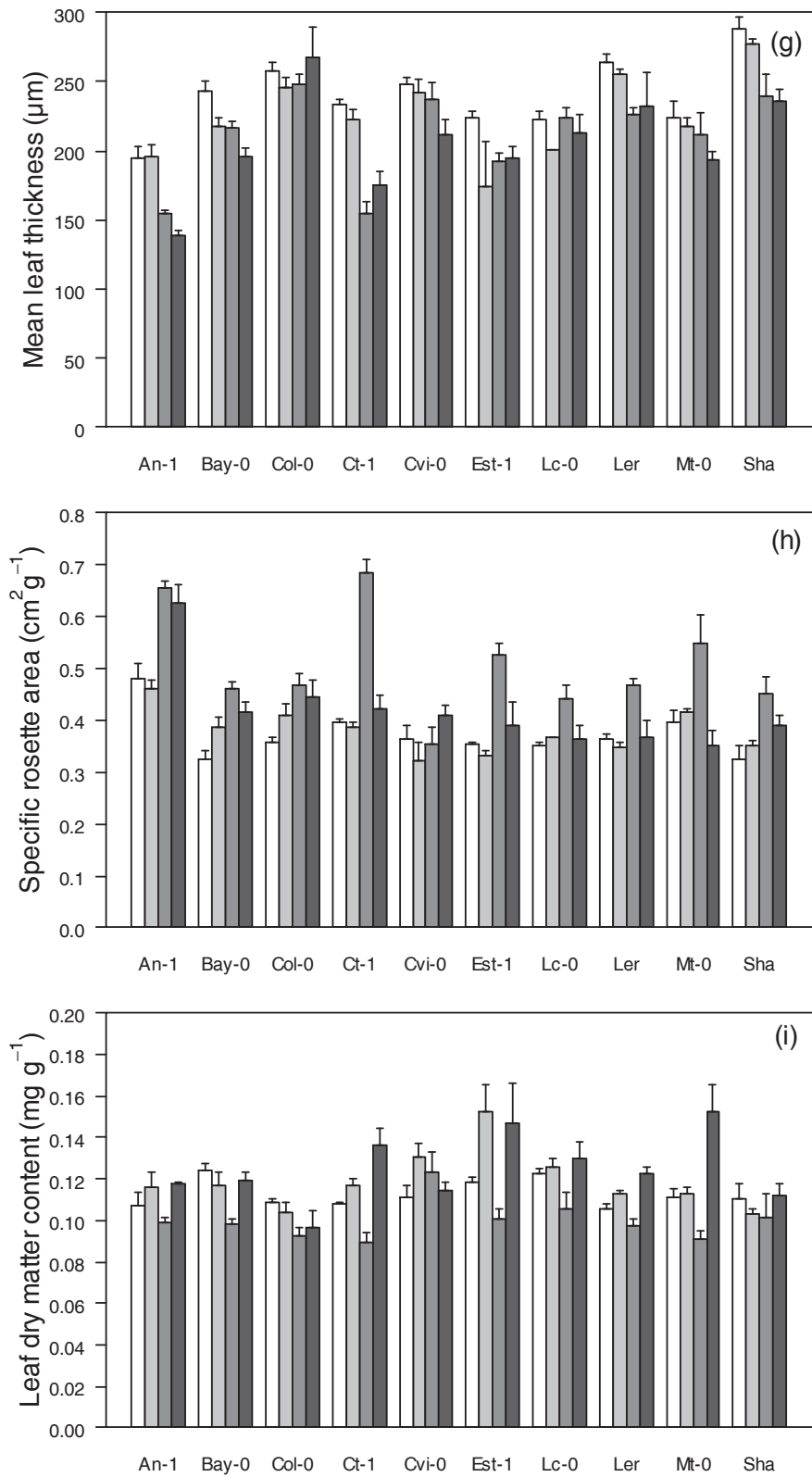


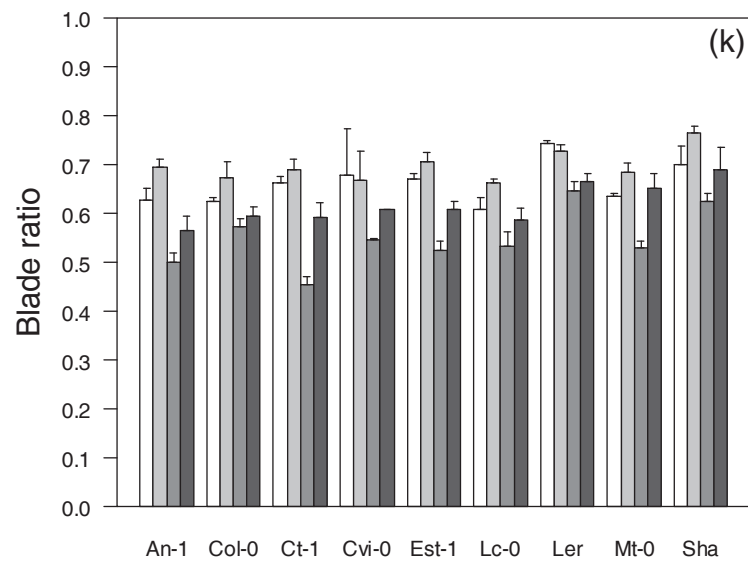
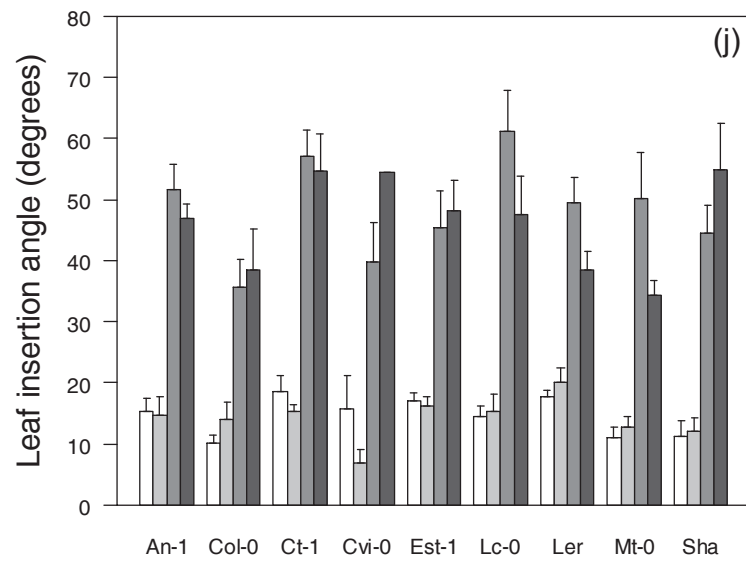


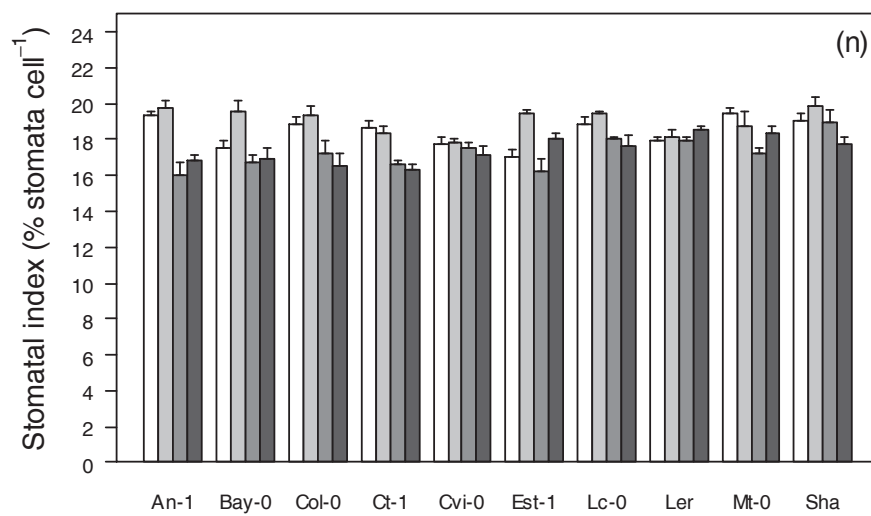
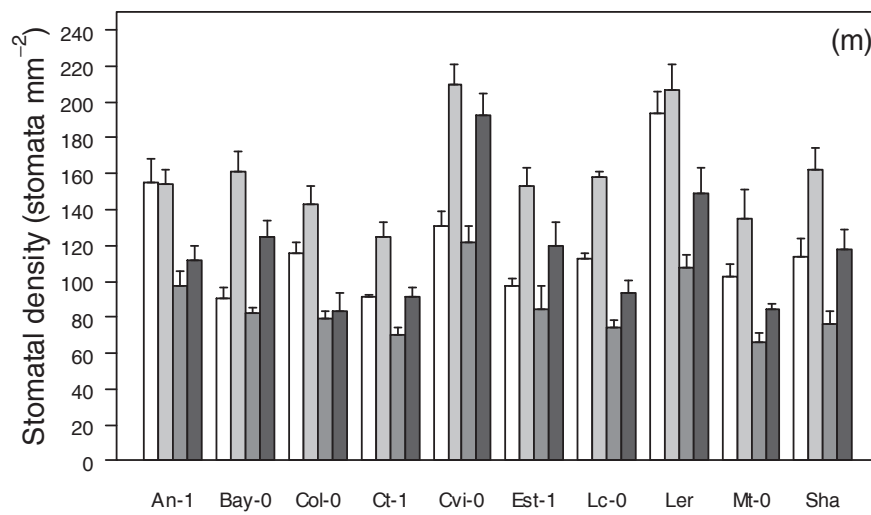
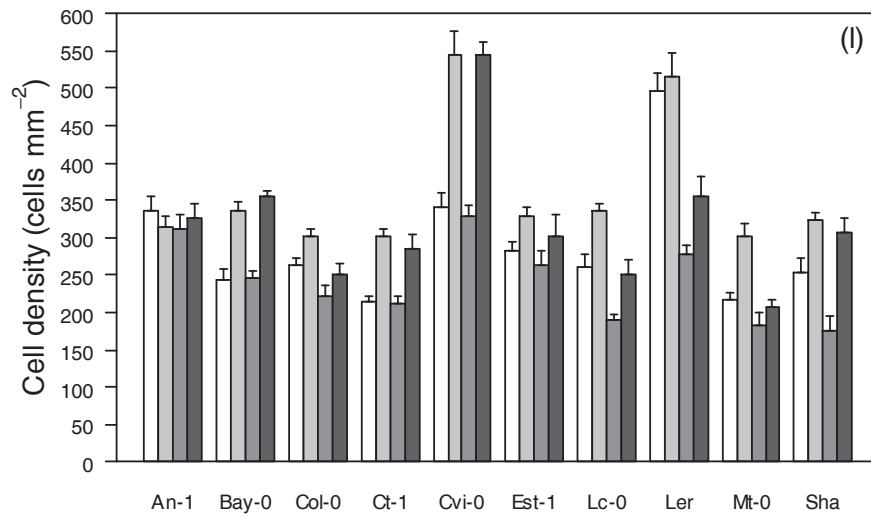
**Figure S2.** Mean trait values by genotypes under control (CT, 20/17°C day/night) and high temperature (HT, 30/25°C day/night), and in well-watered (WW, 35% g H<sub>2</sub>O g<sup>-1</sup> dry soil) and water deficit (WD, 20% g H<sub>2</sub>O g<sup>-1</sup> dry soil) conditions.



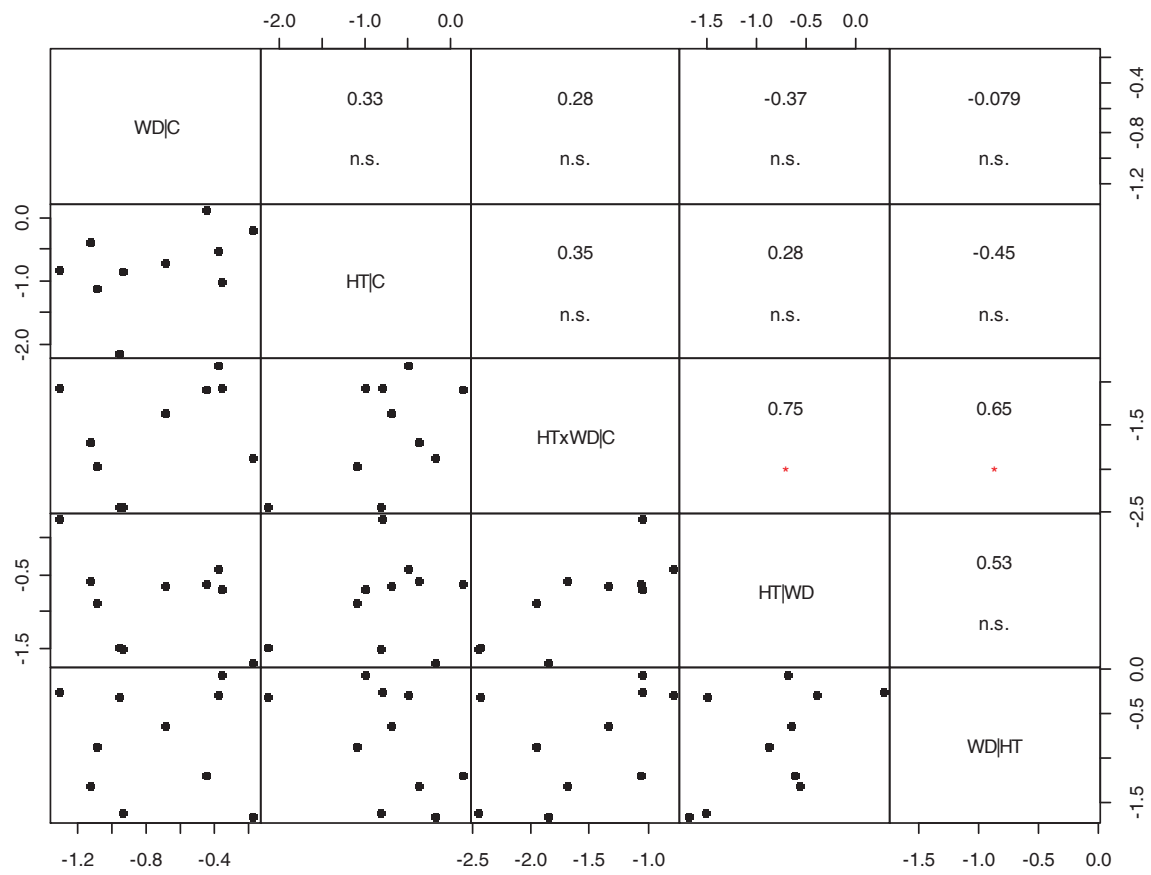




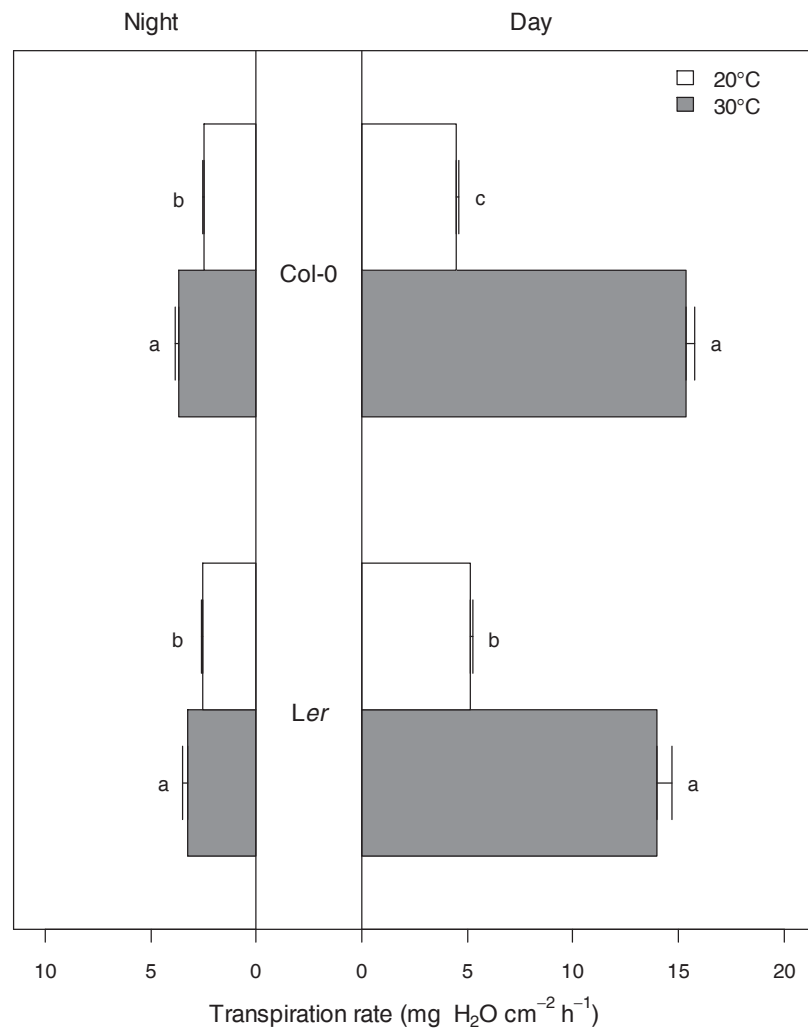




**Figure S3.** Correlation matrix of response ratios (log) for total dry mass.



**Figure S4.** Night and day transpiration rates of Col-0 and *Ler* accessions. Transpiration was determined gravimetrically in plants at bolting stage grown under control (20°C, white) and high (30°C, grey) temperature in well-watered conditions. Bars are means  $\pm$  SE ( $n = 5-10$ ). Different letters indicate significant differences ( $P < 0.05$ ) following Kruskal-Wallis nonparametric tests independently performed for night and day.







## Résumé

Les bactéries promotrices de la croissance des plantes (PGPR) peuvent améliorer la performance et la tolérance des plantes lors de stress environnementaux. *Arabidopsis thaliana* est un modèle de choix pour étudier les mécanismes impliqués dans les interactions plante-bactéries. Nous avons analysé de multiples traits associés à la dynamique de croissance, au développement et la physiologie des végétaux afin d'évaluer les effets de l'inoculation par *Phyllobacterium brassicacearum* STM196, une PGPR isolée de la rhizosphère du colza, sur les réponses d'*A. thaliana* à des stress hydriques de différentes intensités. Grâce à des outils performants de phénotypage, nous avons développé une nouvelle approche d'analyse à haut-débit pour examiner l'implication de STM196 dans les stratégies de résistance des plantes au stress hydrique. Nos résultats montrent pour la première fois que les PGPR peuvent interférer dans les stratégies d'échappement des plantes grâce à des modifications de la croissance et du temps de floraison. De plus, STM196 induit une meilleure résistance au déficit hydrique modéré et une meilleure tolérance à la déshydratation sous une contrainte hydrique sévère. L'inoculation par STM196 peut ainsi représenter une valeur ajoutée aux stratégies de résistance intrinsèques aux plantes, ce qui est illustrée par sa remarquable capacité à promouvoir la survie et la production de biomasse végétale dans des environnements contrastés. Nos résultats soulignent l'importance des interactions plantes-bactéries dans les réponses des plantes à la sécheresse et offrent de nouvelles voies de recherches pour l'amélioration de la résistance à la sécheresse dans les cultures.

**Mots clés :** Bactéries promotrices de la croissance des plantes (PGPR), *Arabidopsis thaliana*, *Phyllobacterium brassicacearum* (STM196), interaction plante-bactérie, déficit hydrique, stratégies de résistance des plantes.

## Abstract

Plant growth promoting rhizobacteria (PGPR) can enhance plant performance and plant tolerance to environmental stresses. *Arabidopsis thaliana* is a useful organism to study the mechanisms involved in plant-PGPR interactions. We analyzed multiple plant traits related to growth dynamics, development and physiology in order to assess the effects of *Phyllobacterium brassicacearum* STM196 strain, isolated from the rhizosphere of oilseed rape, on *Arabidopsis* responses to well-defined soil water availability. Using powerful tools for phenotyping, we developed a new high-throughput analysis to examine the implication of STM196 on plant strategies to cope with water stress. Our results show for the first time that PGPR can interfere in escape strategies of plants through modifications in plant growth and flowering time. Moreover, STM196 induced a better resistance to moderate water deficit and a better tolerance to dehydration under a severe stress. Inoculation by STM196 can represent an added value to plant resistance strategies, as illustrated by its remarkable ability to promote plant survival and biomass production under contrasted environments. Our results highlight the importance of plant-bacteria interactions in plant responses to drought and provide a new avenue of investigations to improve drought resistance in crops.

**Key words:** Plant growth promoting rhizobacteria (PGPR), *Arabidopsis thaliana*, *Phyllobacterium brassicacearum* (STM196), plant-bacteria interactions, water deficit, plant resistance strategies.