Grapevine root growth under water stress and its relationship to root water uptake

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Grapevine root growth under water stress and its relationship to root water uptake

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Titre : La croissance racinaire de la vigne en conditions de sécheresse et sa relation avec l'absorption d'eau racinaire

Résumé : Le sujet de l'adaptation aux changements climatiques est devenu l'un des sujets contemporains les plus importants dans la vigne. Une grande focalisation a été mise sur la compréhension des effets du porte-greffe sur la croissance du scion, l'absorption des nutriments, et la tolérance au stress, dans l'objectif final de développer de nouveaux porte-greffes qui facilitent l'adaptation au changement climatique. L'objectif de cette thèse est d'examiner comment les différences dans la résistance à la sécheresse entre les génotypes peut résulter en de grandes différences dans leur capacité à maintenir leur croissance racinaire en situation de stress. Une meilleure compréhension sur la manière dont la structure, la croissance racinaire et l'absorption d'eau répondent au stress nous permettra de mieux comprendre quels sont les aspects de la physiologie racinaire qui contribuent à la tolérance face à la sécheresse. Des recherches précédentes qui s'étaient focalisées sur l'absorption d'eau racinaire chez la vigne ont suggéré que l'absorption d'eau racinaire pouvait être fortement liée à la vitesse de croissance racinaire instantanée (voir Gambetta *et al.* 2013). Cette observation implique que des différences entre les génotypes dans la résistance face à la sécheresse pourrait largement résulter de leur capacité à maintenir la croissance racinaire en conditions de stress. Deux porte-greffes de vigne avec des capacités contrastées en matière de résistance à la sécheresse, le Riparia Gloire de Montpellier (RGM) et le 110 Richter (110R) ont été sélectionnés pour étudier dans cette thèse. RGM est considéré comme sensible à la sécheresse, tandis que 110R est fortement résistant à la sécheresse (Carbonneau 1985). La thèse a examiné la relation entre la croissance racinaire et la capacité de résistance à la sécheresse en évaluant la vitesse de croissance racinaire, la conductivité hydraulique à travers deux variétés de porte-greffe en conditions de déficit en eau. Le niveau de l'expression des gènes d'aquaporines (via la qPCR et l'ARNseq) et leur contribution à la conductivité hydraulique racinaire ont été analysés dans les radicelles afin d'obtenir une meilleure compréhension sur les mécanismes impliqués dans la régulation de l'absorption de l'eau racinaire et la conductivité hydraulique au cours du développement et en réponse à un manque d'eau.

Le traitement de stress d'eau prolongé a diminué le potentiel hydraulique de la plante. La croissance racinaire individuelle est très hétérogène : bien que le traitement de sécheresse réduise l'élongation racinaire en moyenne, la vitesse de croissance racinaire varie tout de même énormément. Un haut niveau de stress hydrique a réduit significativement la vitesse de croissance racinaire moyenne à la fois pour RGM et 110R. Globalement, la vitesse de croissance racinaire moyenne a montré une tendance réduite au cours du développement de la plante. La température du sol est aussi un facteur qui affecte la croissance racinaire. Pour RGM et 110R, en conditions

de bon arrosage et de stress hydrique, la vitesse de croissance quotidienne moyenne a été positivement corrélée avec la température du sol quotidienne moyenne. En conditions de bon arrosage, des vitesses de croissance racinaires plus importantes ont été constamment observées chez 110R par rapport à RGM, ce qui pourrait être une explication possible de sa meilleure résistance à la sécheresse par rapport à 110R.

La conductivité hydraulique racinaire (Lp_r) a été influencée à la fois par le traitement de stress hydrique et le stade de développement de la plante. Généralement, à la fois chez RGM et 110R, le Lp_r a été significativement réduit en conditions de stress hydrique au stade précoce. Lors des stades moyens et tardifs, aucune différence significative de Lp_r n'a été observée entre les plantes bien arrosées et en conditions de stress. Des modifications de Lpr racinaires individuels en réponse au potentiel de base ($\Psi_{predawn}$) ont aussi été recherchées. Le Lpr a montré une forte chute au début du stress hydrique lorsque $\Psi_{predawn}$ était supérieur à -0,5 MPa. Cependant, avec $\Psi_{predawn}$ devenant plus négatif, i.e. de -0,4 à -2,0 MPa, la gamme des valeurs de Lp_r mesurées dans notre étude est restée constante. Le Lp_r des plantes bien arrosées a aussi diminué bien que leur $\Psi_{predawn}$ ait été maintenue à un haut niveau (< 0,1 MPa) au cours de la période d'expérimentation.

L'abondance de transcription des gènes d'aquaporines en réponse au stress hydrique et en fonction des stades de développement a été analysée à la fois par RT-qPCR (seulement les *Vv*PIPs) et ARN-seq (famille MIP). Une comparaison des données de l'expression des gènes des RT-qPCR et ARN-seq a révélé qu'il existe une bonne correspondance dans les schémas d'expression des gènes pour la majorité des gènes entre ces deux méthodes. Davantage de gènes MIPs ont été surexprimés en condition de faible stress hydrique tandis que davantage de gènes MIPs ont été sous-exprimés en conditions de stress hydrique important. En conditions bien arrosées, une diminution significative de certains gènes *Vv*TIP a été observée au cours du développement en particulier chez 110R.

Nous avons observé des corrélations significatives mais peu claires entre la conductivité hydraulique racinaire et la croissance racinaire ainsi qu'entre le niveau d'expression de certains gènes *Vv*PIP et le taux de croissance racinaire. Cependant, la corrélation entre les relations d'eau racinaire et la vitesse de croissance racinaire semble plus complexe. Potentiellement, avec les résultats de nos analyses ARN-seq, nous pourrions trouver des gènes qui régulent la croissance racinaire en plus des *Vv*PIPs que nous avons analysés, dans le but d'obtenir une compréhension plus globale sur la régulation de la croissance et du développement racinaires en conditions à la fois de contrôle et de stress hydrique.

Mots clés : Vigne, porte-greffe, stress hydrique, croissance racinaire, conductivité hydraulique racinaire, aquaporines

Title: Grapevine root growth under water stress and its relationship to root water uptake

Abstract: The subject of adaptation to climate change has become one of the most important contemporary topics in grapevine. Much focus has been placed on the understanding of rootstocks effects on scion growth, nutrient uptake, and tolerance to stress, with the ultimate goal of developing novel rootstocks that facilitate adaptation to a changing climate. The purpose of this thesis is to examine how differences in drought resistance between genotypes could result largely from differences in their ability to maintain root growth under stress. A better understanding of how root structure, growth, and water uptake respond to stress will allow us to better understand what aspects of root physiology contribute to drought tolerance. Previous research focused on root water uptake in grapevine suggested that root water uptake could be tightly coupled to a root's instantaneous rate of growth (see Gambetta *et al.* 2013). This observation implies that differences in drought resistance between genotypes could result largely from their ability to maintain root growth under stress. Two grapevine rootstocks with contrasting drought resistance capacity, Riparia Gloire de Montpellier (RGM) and 110 Richter (110R), were selected to study in this thesis. RGM is considered as sensitive to drought, while 110R is highly resistant to drought (Carbonneau 1985). The thesis examined the relationship between root growth and drought resistant capacity by assessing root growth rate, hydraulic conductivity across two rootstock varieties subjected to water deficit. The role of aquaporin gene expression (via qPCR and RNAseq) and their contribution to root hydraulic conductivity were analyzed in fine roots in order to obtain a better understanding on the mechanisms involved in the regulation of root water uptake and hydraulic conductivity across development and in response to water deficit.

Prolonged water stress treatment decreased plant water potential. Individual root growth is very heterogeneous, although drought treatment reduces root elongation on average, individual root growth rate still varies enormously. High level of water stress significantly reduced average root growth rate for both RGM and 110R. Globally, average root growth rate showed a decreased trend over plant development. Soil temperature is also a factor that affects root growth. For both RGM and 110R, under both well-watered and water-stressed conditions, average daily root growth rate was positively correlated with average daily soil temperature. Under well-watered conditions, higher root growth rates were constantly observed in 110R compared to RGM, which could be one possible explanation for the higher capacity in drought resistance of 110R.

Root hydraulic conductivity (Lp_r) was influenced by both water stress treatment and plant developmental stage. Generally, for both RGM and 110R, Lp_r was significantly reduced under water stress in early stage. In mid and late stages, no significant differences in Lp_r were observed

between well-watered and water-stressed plants. Changes in individual root Lp_r in response to pre-dawn leaf water potential ($\Psi_{predawn}$) were investigated as well. Lp_r showed a fast drop in the beginning of water stress treatment when $\Psi_{predawn}$ was higher than -0.5 MPa. However, with $\Psi_{predawn}$ getting more negative, e.g. from -0.4 MPa to -2.0 MPa, the range of Lp_r values measured in our study maintained constant. Lp_r of well-watered plants decreased as well even though their $\Psi_{predawn}$ was maintained at a high level (< 0.1 MPa) during the period of the experiment.

Transcript abundances of aquaporin genes in response to water stress and developmental stages were analyzed via both RT-qPCR (only *Vv*PIPs) and RNA-seq (MIP family). Comparison of gene expression data from RT-qPCR and RNA-seq revealed that there is a good correspondence in gene expression patterns for the majority of genes between these two methods. More MIP genes were up-regulated under low level of water stress while more MIP genes were down-regulated under high level of water stress. Under well-watered conditions, significant down-regulation of certain *Vv*TIP genes were observed over development particularly in 110R.

We observed some significant but noisy correlations between root hydraulic conductivity and root growth as well as between the expression level of some *Vv*PIP genes and root growth rate. However, the correlation between root water relations and root growth rate seems to be more complex. Potentially, with the results from our RNA-seq analysis, we could find genes that regulate root growth in addition to these *Vv*PIPs we analyzed and obtain a more comprehensive understanding on the regulation of root growth and development under both control and water-stressed conditions.

Keywords: Grapevine, rootstock, water stress, root growth, root hydraulic conductivity, aquaporins

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The whole of science is nothing more than a refinement of everyday thinking.

Albert Einstein

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Table of Content

Abbreviations

ABA: Abscisic acid
ANOVA: Analysis of variance
cDNA: Complementary DNA
CT: Control, well-watered
cv.: Cultivar
DNA: Deoxyribonucleic acid
ESTs: Expressed Sequence Tags
FPKM: Fragments Per Kilobase of exon per Million
GLM: Generalized linear model
GHS: Greenhouse gas
Lp_r: Root hydraulic conductivity
Mhl: Million of hectoliters
MIPs: Major Intrinsic Proteins
MPa: Megapascal
mRNA: messager RNA
Mt: Million of tons
NIPs: Nodulin26-like Intrinsic Proteins
PAR: Photosynthetic active radiation
PIPs: Plasma Membrane Intrinsic Proteins
RT-qPCR: Real Time Quantitative Polymerase Chain Reaction
QTL: Quantitative Trait Loci
RGM: *Vitis riparia* cv. Gloire de Montpellier
RNA: Ribonucleic acid
RNA-seq: RNA-sequencing
SAGE: Serial Analysis of Gene Expression
SIPs: Small Basic Intrinsic Proteins
TF: Transcription factors
TIPs: Tonoplast Intrinsic Proteins
Tukey's HSD: Tukey's honest significant difference test
110R: 110 Richter (*Vitis berlandieri* x *Vitis rupestris*)
WS: Water stress
Ψ: Water potential
Ψ_{midday}: Mid-day stem water potential
$\Psi_{predawn}$: Pre-dawn leaf water potential

List of Figures

List of Tables

General introduction

Grapevines are a widely cultivated and economically important perennial fruit crop in many countries across the world. Some countries mainly cultivate wine grape varieties and are specialized in wine production such as France, Italy, Spain, and Argentina, while some other countries are more focused on table and dried grapes production, such as China, India and Turkey (O.I.V. 2017, Figure 1A). In 2016, a total of 75.8 million of tons (mt) of grapes were produced worldwide, in which almost half of them were used for wine production, making 267 million of hectoliters (mhl) of wine, with Italy, France, and Spain being the most important wine producing countries (O.I.V. 2017, Figure 1B).

The topic of climate change is not new to us. Nowadays, sufficient evidence has shown that global climate change is a serious problem facing humanity. According to the latest records, average global and ocean temperature anomalies have increased from 0.02 °C in the 1950s to 0.77 °C in the 2010s (Figure 2A), precipitation anomalies displayed significant variations around the world (Figure 2B), and abnormal climate events have been observed across the world (Figure 2C). The expansion of arid areas has increased in many land regions based on historical data of precipitation, streamflow, and drought indices (Dai 2013), and drought has been a widespread issue under climate change (Dai 2011). The oceans are also warming (temperature has risen by 0.10 °C from the surface to a depth of 700 m from 1961 to 2003) and the global average sea level is rising with an average speed of 1.7 ± 0.5 mm/year for the 20th century (Bindoff *et al.* 2007). Scientific evidence suggests that global warming is very likely anthropogenic (Rosenzweig 2008) and the concentration of atmospheric greenhouse gas (GHS, e.g. CO_2, CH_4, N_2O) has increased since the 1950s (Figure 3).

Evidence has been shown that climate change is driving a global biological response (Brown *et al.* 2016) as indicted by phenology and distribution shifts in a large number of marine (Poloczanska *et al.* 2013) and terrestrial (Parmesan and Yohe 2003) species. Plant phenology has been known to be sensitive to year-to-year variability in weather (Richardson *et al.* 2013) and climatic shifts together with the change of seasons predominantly drive the annual initiation of phenological events, rather than intrinsic controls (Badeck *et al.* 2004). In the context of climate change, early onset of vegetation activity in spring and an overall extension in the length of the active growing season have been observed (Badeck *et al.* 2004, Linderholm, 2006, Yang *et al.* 2017). In terms of the movement of species distribution, it is generally expected that species track the shifting climate and shift their own distribution poleward in latitude and upward in elevation (Walther *et al.* 2002). However, during the rapid climate changes in the past, differential movements have been shown between species, which could result in a disruption of the connectedness among many species in current ecosystems (Root *et al.* 2003).

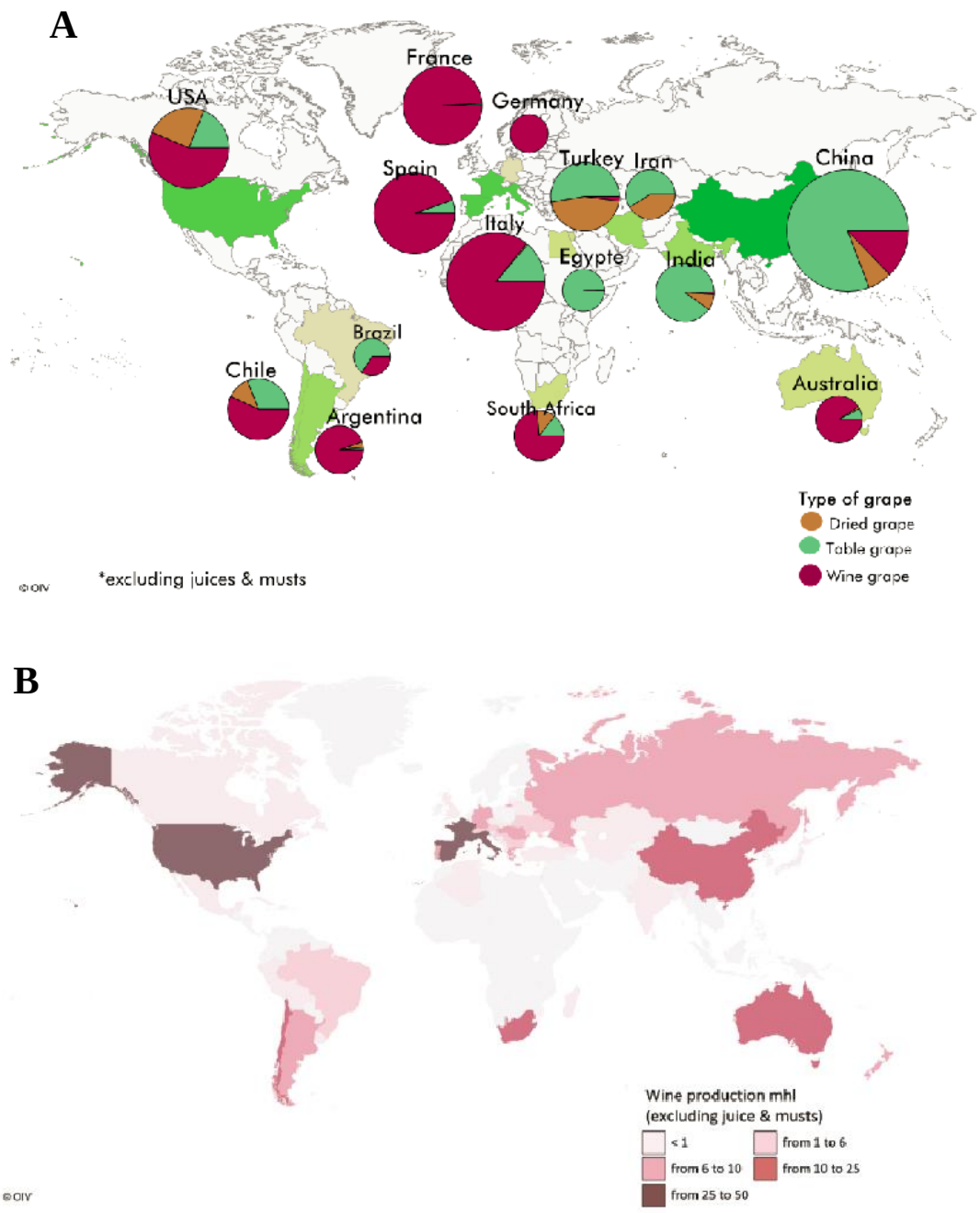

Figure 1 Major grape producers by type of products (A) and major wine producers (B) in the world in 2016 (From O.I.V. 2017)

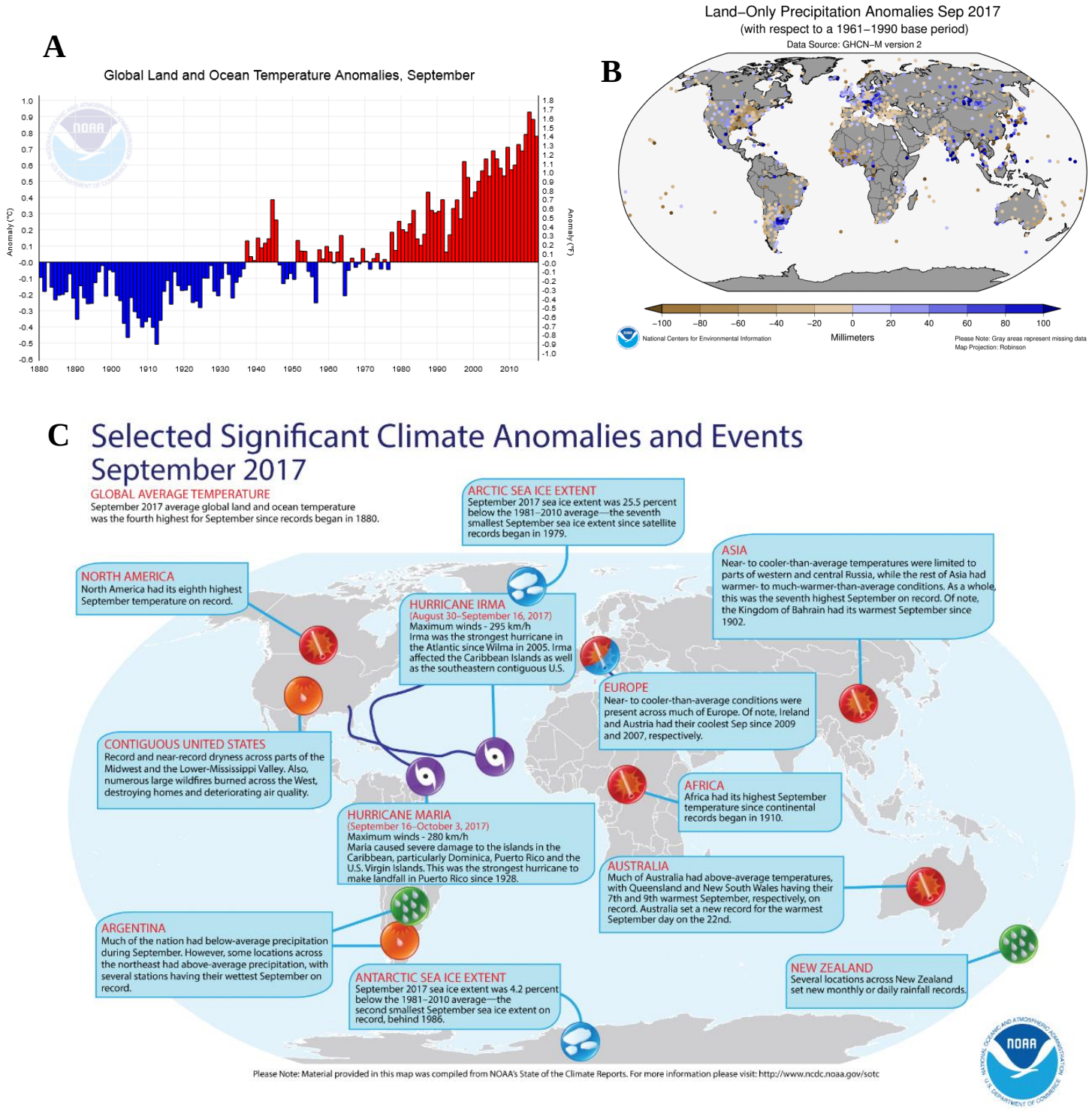

Figure 2 Global climate anomalies. A, Global Land and Ocean Temperature Anomalies, September 2017. B, Global land-only precipitation anomalies, September 2017. C, Selected significant global climate anomalies and events, September 2017. Source: NOAA National Centers for Environmental information, Climate at a Glance: Global Time Series, published October 2017, retrieved on November 4, 2017 from http://www.ncdc.noaa.gov/cag/

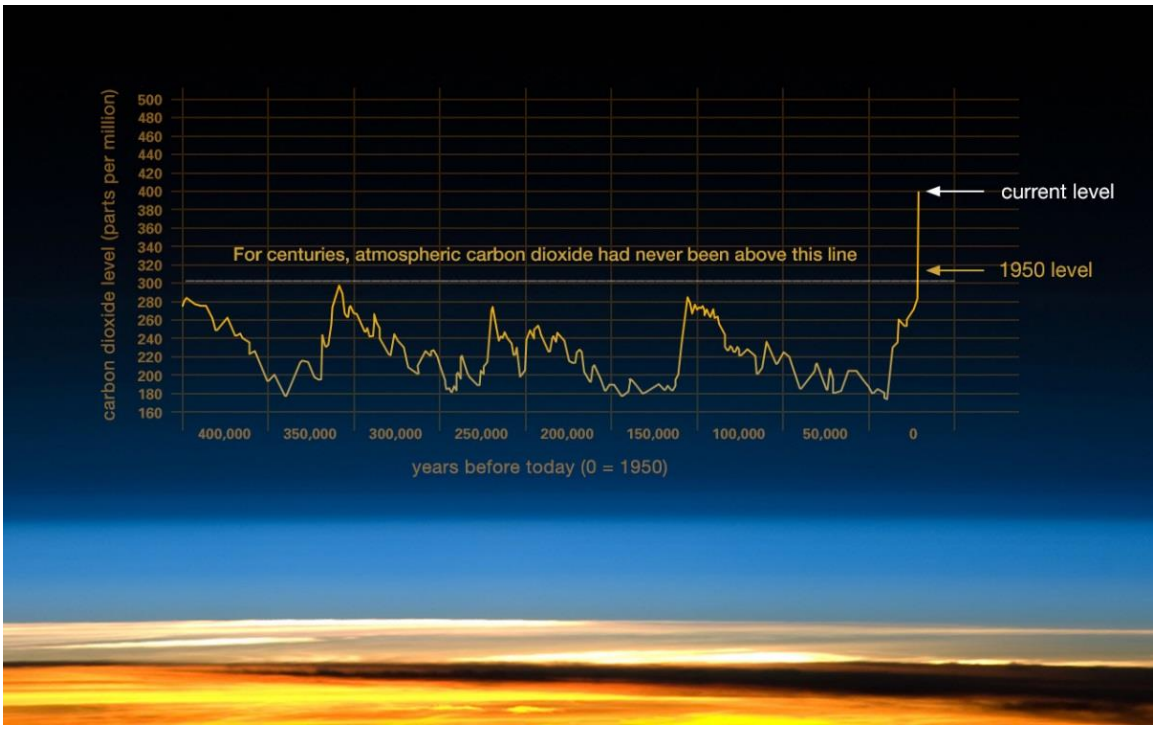

Figure 3 Changes of atmospheric CO_2. This graph, based on the comparison of atmospheric samples contained in ice cores and more recent direct measurements, provides evidence that atmospheric CO_2 has increased since the Industrial Revolution. (Credit: Vostok ice core data/J.R. Petit *et al.*; NOAA Mauna Loa CO_2 record.) Source: https://climate.nasa.gov/evidence/

Grapevine growth and development depend on its growing environment, and so does the quality of grape berries. In this sense, the quality of wine is determined by the environment where it comes from. The influences of recent and long-term climate changes on grapevine growth and development as well as on berry quality have been examined by many researchers (Kenny GJ and Harrison1992, Tate 2001, Jones *et al.* 2005, Holland and Smit 2010, Jones and Webb 2010, Schultz and Jones 2010). Climate change is increasing the focus and investment on the development of more drought resistant rootstock and scion varieties with the potential to maintain yields, increase water conservation through reducing the need for irrigation, and/or protect vines from long term damage resulting from drought. Knowledge of the mechanisms by which rootstocks influence plant behavior can better inform plant material selection and is a critical component in the development of new rootstock varieties (Zhang *et al*. 2016).

Objectives of the thesis

The subject of adaptation to climate change has become one of the most important contemporary topics in grapevine. Much focus has been placed on the understanding of rootstocks effects on scion growth, nutrient uptake, and tolerance to stress, with the ultimate goal of developing novel

rootstocks that facilitate adaptation to a changing climate. A better understanding of how root structure, growth, and water uptake respond to stress will allow us to better understand what aspects of root physiology contribute to drought tolerance. Previous research focused on root water uptake in grapevine suggested that root water uptake could be tightly coupled to a root's instantaneous rate of growth (see Gambetta *et al.* 2013). This observation implies that differences in drought resistance between genotypes could result largely from their ability to maintain root growth under stress. Two grapevine rootstocks with contrasting drought resistance capacity, Riparia Gloire de Montpellier (RGM) and 110 Richter (110R), were selected to study in this thesis. RGM is considered as sensitive to drought, while 110R is highly resistant to drought (Carbonneau 1985). The thesis examined the relationship between root growth and drought resistant capacity by assessing root growth rate, hydraulic conductivity across two rootstock varieties subjected to water deficit. The level of aquaporin gene expression (via RT-qPCR and RNAseq) and their contribution to root hydraulic conductivity were analyzed in fine roots in order to obtain a better understanding on the mechanisms involved in the regulation of root water uptake and hydraulic conductivity across development and in response to water deficit.

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Zhang L, Marguerit E, Rossdeutsch L, Ollat N, and Gambetta GA. The influence of grapevine rootstocks on scion growth and drought resistance. Theoretical and Experimental Plant Physiology. 2016, 28(2), 143-157

Reynolds AG, Baker L, **Zhang L**, Jasinski M, Di Profio F, Kogel S, Pickering G. Impacts of natural yield variances on wine composition and sensory attributes of *Vitis vinifera* cvs. Riesling and Cabernet franc. Canadian Journal of Plant Science. 2017 Accepted with revisions

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Wong D, **Zhang L**, Merlin I, Castellarin SD, Gambetta GA. Structure and transcriptional regulation of the major intrinsic protein gene family in grapevine. BMC Genomics. 2018 Submitted

Zhang L, Merlin I, Ollat N, Gambetta GA. Developmental and drought induced coordination of fine root growth rate and hydraulic conductivity in grapevine. 2018, manuscript in preparation

Chapter 1 Literature review. Part I. A review: The influence of grapevine rootstocks on scion growth and drought resistance

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Abstract

Grapes are a widely cultivated and economically important crop. Climate change is increasing the focus and investment on the development of more drought resistant varieties. However, markets often dictate specific grape varieties that can be grown and sold. Thus growers are increasingly interested in conferring particular traits of interest (e.g., drought tolerance) through grafting onto rootstocks. A major goal is to develop rootstocks that can influence scion growth and productivity under drought; particularly those that can increase water conservation through reducing the need for irrigation while ameliorating negative impacts on yields. Growers and scientists recognize that rootstocks have a profound influence on vine physiology (e.g., stomatal conductance, photosynthesis, water status), productivity (e.g., growth, fruit yields, fruit composition), and drought resistance. The challenge is to better understand the exact mechanisms through which rootstocks manifest these effects and thus build the knowledge necessary to drive the development of rootstocks with predictable effects on the scion. The aim of this review is to explore our current understanding of the mechanisms by which grapevine rootstocks influence scion growth and stress response; specifically focused on the integration of vine growth and productivity under water deficit.

Keywords: Vigor, Water deficit, Hormone signaling, Hydraulic signaling, Abscisic acid, Climate change

1.1 Introduction

Grapes are one of the most valuable perennial crops in the world. In the context of global climate change there is increasing investment in the development of more drought resistant plant material (i.e. rootstock and scion varieties). New plant material may have the potential to maintain yields, increase water conservation through reducing the need for irrigation, and/or protect vines from long term damage resulting from drought. However, local and globalmarkets often constrain the type of grape varieties that can be grown and thus growers are increasingly interested in

Table 1.1 Traditional classifications of drought resistance and vigor rating of a variety of common rootstocks

Name	Parentage	Drought resistance classification			Vigor rating
		Samson and Casteran (1971)	Fregoni *et al.* (1978)	Carbonneau (1985)	Samson and Casteran (1971) and Cordeau (2002)
110R	*Rupestris x Berlandieri*	High	High	Highly resistant	Vigorous - highly vigorous
140Ru	*Rupestris x Berlandieri*	Medium	High	Highly resistant	Highly vigorous
44-53	*Rupestris x Cordifilia x Riparia*	High	High	Highly resistant	Medium - vigorous
1103P	*Rupestris x Berlandieri*	High	High	Resistant	Highly vigorous
196-17C	*Vinifera x Rupestris x Riparia*		High (Pongrazc 1983)		Highly vigorous
SO4	*Riparia x Berlandieri*	Low	Low	Resistant	Vigorous - highly vigorous
Dogridge	*Rupestris x Candicans = V. Champini*		Low to medium (Southey 1992)		Highly vigorous (Hardie and Cirami, 1990)
Ramsey			Medium (Pongrazc 1983)		Highly Vigorous (Hardie and Cirami 1990)
99R	*Rupestris x Berlandieri*	Medium	Medium	Sensitive	Highly vigorous
3309C	*Riparia x Rupestris*	High	Low	Sensitive	Medium
420A	*Riparia x Berlandieri*	Low	Low	Sensitive	Medium—vigorous
Fercal	*Berlandieri x Vinifera*	Medium		Sensitive	Medium—vigorous
5BB	*Riparia x Berlandieri*	Low	Low	Sensitive	Vigorous - highly vigorous
161-49 MGt	*Riparia x Berlandieri*	Low	Medium	Sensitive	Medium—vigorous
41B	*Berlandieri x Vinifera*	Medium	High	Sensitive	Medium—vigorous
Rupestris du Lot	*Rupestris*	Low	Low	Sensitive	Vigorous - highly vigorous
101-14 MGt	*Riparia x Rupestris*	Low	Low	Very sensitive	Weak - medium
Riparia Gloire de Montpellier	*Riparia*	Low	Low	Very sensitive	Weak
333EM	*Berlandieri x Vinifera*	High	Medium	Very sensitive	Vigorous

Drought resistance classifications are primarily taken from the three works, but exceptions are noted in parentheses

conferring particular traits of interest (e.g., drought tolerance) through grafting. Knowledge of the mechanisms by which rootstocks influence plant behavior can better inform plant material selection and is a critical component in the development of new rootstock varieties.

Grapevines are generally woody lianas (i.e. vines) (Mullins *et al.* 1992; Keller 2015). Cultivated grapevines are found predominately in temperate climate zones. In many wine producing regions, grapevines experience seasonal periods of drought (Mederano 2003; Chaves *et al.* 2010). But unlike other crop plants, wine grapes are relatively resistant to drought and moderate levels of water deficit, despite negative impacts on fruit yields, are widely recognized as having positive effects on fruit quality especially with respect to wine (Mederano 2003; Deluc *et al.* 2009; Van Leeuwen *et al.* 2009).

Grapevines are almost exclusively propagated vegetatively from woody cuttings, and the vast majority (> 80 %) are grafted (Ollat *et al.* 2015). Historically, grafting became prominent after the introduction of phylloxera to Europe in the late 19th century. Grafting allows for the combination of phylloxera resistant rootstocks derived from American Vitis species and the superior fruit quality of the Eurasian species *Vitis vinifera* (Keller 2015). Most rootstock varieties are interspecific hybrids of the American species*: Vitis riparia*, *Vitis rupestris*, and *Vitis berlandieri*. Although the level of phylloxera resistance is a critical trait, rootstocks are also valued for their resistance to other pathogens, to drought, to water-logging, their adaptation to different soil types, as well as their influence on scion vigor and grape composition (Mullins *et al.* 1992; Granett *et al.* 2001; Jackson 2008; Keller 2015). When selecting rootstocks typically a combination of these criteria is taken into account based on the particular environmental conditions (i.e. soil and climate) of the vineyard (Granett *et al*. 2001).

Vitis riparia, *Vitis rupestris*, and *Vitis berlandieri* are each adapted to specific environments resulting in hybrids exhibiting a wide variety of traits. *Vitis riparia* is well adapted to relatively wet environments with a shallow root system, *Vitis rupestris* is adapted to gravel and sandy soils with a deep rooting growth habit, while *Vitis belandieri* is native to calcareous high pH soils. Some selections from *Vitis riparia* (e.g. Riparia Gloire de Montpellier; RGM) and *Vitis rupestris* (e.g. Rupetris, St. George) are directly used as rootstocks but none from *Vitis belandieri* due to the fact that rooting is difficult from cuttings. Numerous hybrids across these three species have been developed by breeders and they play an important role in today's viticulture being used as the vast majority of rootstocks (Ollat *et al.* in press; Cousins 2005). However, some parts of Australian and Chile are phylloxera-free and grapevines are own-rooted (Mullins *et al.* 1992; Jackson 2008; Keller 2015).

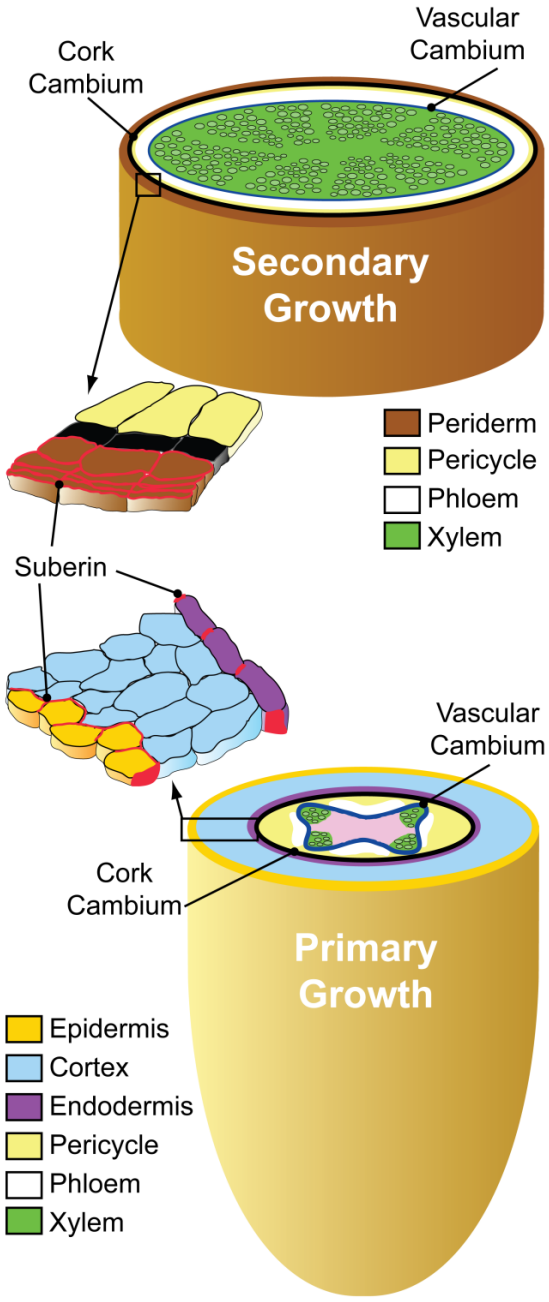

Figure 1.1 The structure of grapevine roots exhibiting primary (below) and secondary (above) growth. Primary growth results in roots consisting of the exodermis, cortex, endodermis, pericycle, and xylem and phloem tissues. As a root matures secondary growth is initiated at the vascular cambium, producing secondary xylem and phloem, and the cork cambium, generating the periderm. The original exodermis, cortex and endodermis are lost in the mature root (above). Roots have suberized structures (red) that can potentially limit the movement of water and solutes through the cell wall space (i.e. apoplast). During primary growth these structures are found in the exodermis and endodermis, and during secondary growth the periderm.

In grafted grapevines, rootstocks make up part of the trunk and the root system. The root system anchors the plant, is responsible for water and nutrient uptake, and is a source for many plant hormones (Richards 1983; Keller 2015). Additionally, the root system is the location of stored carbohydrate and nutrient reserves that promote and maintain root and shoot growth in the beginning of the growing season and under stress. In contrast to plants grown from seeds where the primary root develops from the hypocotyl of the embryo, the grapevine root system is initiated from adventitious roots of woody cuttings. As a woody perennial plant, the development of grapevine roots is comprised of both primary and secondary growth. The young fine roots of grapevine are analogous to herbaceous roots, consisting of epidermis, cortex, endodermis, pericycle, and xylem and phloem tissues (Figure 1.1) (Richards 1983; Keller 2015). As a root matures secondary growth is initiated as the vascular cambium produces secondary xylem and phloem, and the cork cambium (originating from the pericycle) generates the periderm. The original epidermis, cortex and endodermis are lost in the mature root (Figure 1.1). The development of the root system is extremely plastic and root system architecture is regulated to make the best use of accessible resources, in reaction to exogenous biotic and abiotic factors, and to adapt to a changing environment (Smart *et al.* 2006; Bauerle *et al.* 2008; Hochholdinger and Zimmermann 2009; Eshel and Beeckman 2013).

1.2 The influence of rootstock on scion growth and fruit composition

The scion depends on the rootstock for water and mineral nutrients, while the rootstock relies on the scion for photosynthetic assimilates (Kocsis *et al.* 2012). It is obvious that the scion variety determines fruit composition (i.e. berry size, yield, and quality parameters) in grafted plants, but rootstocks can drastically influence and alter these characteristics as well (Davis *et al.* 2008). Thus, the adoption of rootstocks in wine grape production provides an opportunity for growers to manipulate and change varietal traits to improve grape and wine quality without genetically modifying the scion (Jones *et al.* 2009).

Studies have been carried out with different combinations of rootstock and scion, under both field and potted conditions. Synthesizing the published literature on rootstock effects is challenging because there is little consistency in the combinations of rootstocks studied. Nevertheless, there is overlap between some studies and differences in scion, site, climate, and experimental design can allow for a robust assessment of rootstock effects. In theory, if a rootstock has a robust effect on a particular aspect of scion growth it should hold up under different environmental conditions, and even different scions (at least relative other rootstocks). This forms the foundation of rootstock selection.

Rootstocks modify the rate of scion growth over various time scales. Within season, scions grafted onto different rootstocks exhibit different rates of growth both in pots and in the field (e.g. Tardáguila *et al.* 1995; Grant and Matthews 1996; Nikolaou *et al.* 2000; Paranychianakis *et al.* 2004; Tandonnet *et al.* 2008; Tandonnet *et al.* 2010; Ollat *et al.* 2013). For example, Grant and Matthews (1996) grafted Cabernet Sauvignon and Chenin blanc on four different rootstocks

(Freedom, AXR#1, St. George, and 110R) and observed differences in shoot growth after just 10 days in potted vines. Differences in shoot growth were also observed by Cookson et al (2012) comparing Cabernet Sauvignon grafted onto the RGM and 1103P rootstocks. Rootstocks can also influence scion growth over multiple seasons. Ollat *et al.* (2003) conducted a study across 25 years investigating the influences of RGM, 101-14MGt, and SO4 on Cabernet Sauvignon. They found that there were strong differences in pruning weight, shoot growth rate, and biomass allocation among rootstocks.

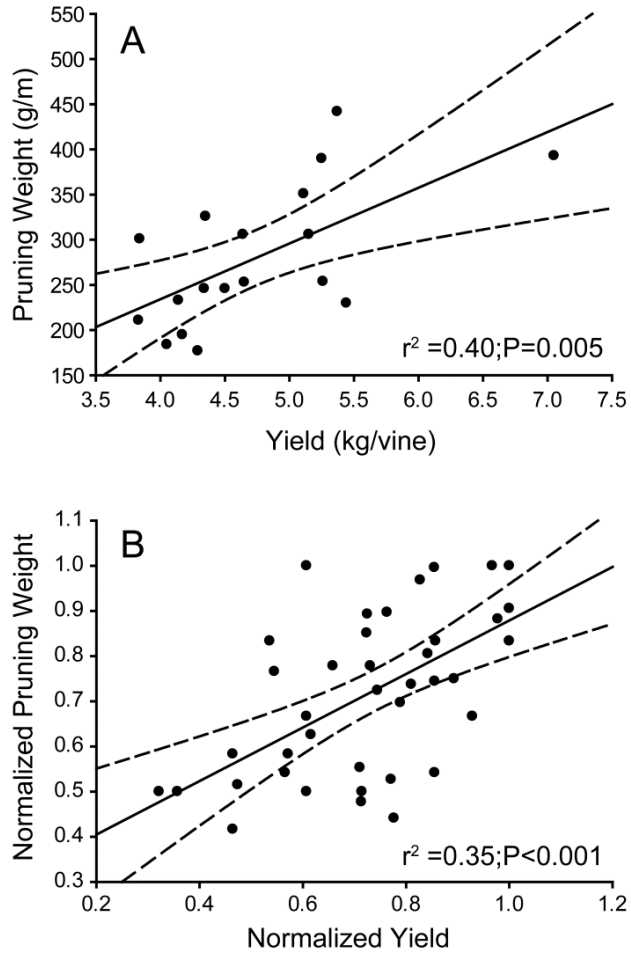

Figure1.2 Examples of the rootstock effect on vigor and yield. A, Results of Keller *et al.* (2012) where yield and pruning weights are reported as 3 year averages of three *Vitis vinifera* varieties (Merlot, Syrah, and Chardonnay) own-rooted or grafted to different rootstocks (5C, 140Ru, 1103P, 3309C, and a new rootstock 101CU). B, Combine meta-analysis of the results of Nikolaou *et al.* (2000), Keller *et al.* (2012) and Kidman *et al.* (2013). Yield and pruning weights were normalized within each study expressed as a ratio to the maximum yield or pruning weight reported for each study (e.g., yieldnormalized = yield/yieldmaximum). R^2, P values, and 95 % confidence intervals are presented for both plots.

Some rootstocks consistently increase vegetative and reproductive growth with respect to other rootstocks. For example, Paranychianakis *et al.* 2004 found that when a sultana (*Vitis vinifera* L.) variety was grafted onto 41B, 1103P, and 110R, 41B produced greater leaf area and higher yields. Similarly, Nikolaou *et al.* (2000) studied the effects of various rootstocks, 420A, 110R, 99R, 41B, Kober 5BB, 8B Teleki, 1103P, 31R and 3309C, on the growth patterns of Thompson Seedless (*Vitis vinifera* L.), finding 41B had the greatest pruning weight and yield.

More generally, works have found that there is a positive correlation between vigor and yield with respect to rootstocks (Walker *et al.* 2002; Jones *et al.* 2009, Tandonnet *et al.* 2012). Several additional studies have reported both yield and pruning weight values for scions grown on various rootstocks but did not correlate them with each other (Nikolaou *et al.* 2000; Keller *et al.* 2012; Kidman *et al.* 2013). Keller *et al.* (2012) carried out a field trial with three *Vitis vinifera* varieties (Merlot, Syrah, and Chardonnay) own-rooted or grafted to different rootstocks (5C, 140Ru, 1103P, 3309C, and a new rootstock 101CU) and our re-analysis of their data revealed this positive relationship as well (Figure 2A). When we carried out a global meta-analysis of the normalized yield and pruning weights reported across all three studies (Nikolaou *et al.* 2000; Keller *et al.* 2012; Kidman *et al.* 2013) we observed the same significant correlation (Figure 1.2 B).

Rootstocks affect fruit yield by acting on distinct yield components such as bud fertility, fruit set, and berry size. The work of Kidman *et al.* (2013) suggested that rootstocks impacted scion fruitfulness and fruit set, however these influences varied between scion genotypes. For Merlot, fruit set was higher when grafted to rootstocks, but this was not the case for Cabernet Sauvignon. In contrast, Keller *et al.* (2012) found that rootstocks generally did not have impacts on fruit set, but instead differences arose through differences in cluster number (i.e. bud fertility) and berry size although rootstock differences were small in comparison to seasonal variation. Similarly, they pointed out that the effect of rootstock on yield formation depended on the scion genotype. Other studies have demonstrated rootstock effects on set. For example, Paranychianakis *et al.* (2004) reported that rootstock difference in yield resulted from both differences in berry weight and in the number of berries.

The influence of rootstock on fruit composition is extremely variable. Ollat *et al.* (2003) found that anthocyanin and glycosylate contents were modified by rootstocks, resulting in higher concentrations in Carbernet Sauvignon fruit grown on RGM when compared to 101-14MGt and SO4. The same study found differences in sugar concentrations although other studies have found the opposite (i.e. no effect on sugar concentration; Nikolaou *et al.* 2000; Paranychianakis *et al.* 2004; Harbertson and Keller 2012; Keller *et al.* 2012). Several studies report an effect of rootstock on pH and/or titratable acidity (Paranychianakis *et al.* 2004; Harbertson and Keller 2012; Keller *et al.* 2012). Koundouras *et al.* (2009) compared two rootstock genotypes (1103P and SO4) showing differences in soluble solids (greater for SO4) but not in anthocyanins and total skin polyphenol concentrations. Harbertson and Keller (2012) reported that rootstock

differences resulted in few significant differences in grape and wine composition suggesting that the dominant factors affecting fruit and wine composition was the scion and the season.

In general, studies clearly demonstrate that scion development and fruit composition are modified by rootstock genotype however they do not always agree with respect to the relative effects of specific rootstocks. Rootstock effects on fruit composition may be linked to an effect on yield or through direct effects on the fruit itself and both are highly dependent on other factors such as seasonal variability in climate. Some authors have suggested that even though rootstocks do indirectly influence scion development, the genotype of scion is still the determinant factor (Tandonnet *et al.* 2010; Keller *et al.* 2012). It is likely that the interaction between scion and rootstock outweighs the impact of rootstock alone (Tandonnet *et al.* 2010).

1.3 Possible mechanisms involved in rootstock-scion interaction

In grafted plants there is integration between two genotypes, rootstock and scion, and the interaction and communication between them is still poorly understood. Better understanding concerning the mechanisms involved in rootstock-scion communication is critical in improving management strategies and grafting technologies (e.g. Cookson *et al.* 2013; Cookson *et al.* 2014). Root systems from different rootstocks differ in terms of their ability to take up water, mineral nutrients, as well as hormone production (Skene and Antcliff 1972; Carbonneau 1985; Ruhl 1991; Brancadoro *et al.* 1993; Ezzahouani and Williams 1995; Nikolaou *et al.* 2000), but how do these differences in water and nutrient uptake potential contribute to rootstock effects? What role does hormonal signaling play in rootstock-scion communication? Can rootstocks alter the gene expression of scion? Is there an exchange of genetic material between the rootstock and scion? These questions speak to the multiple mechanisms through which rootstocks can influence scion growth.

1.3.1 Formation of the rootstock-scion graft union

After grafting, the close contact between the cut surfaces of rootstock and scion leads to the formation of the graft union. Compatibility between rootstock and scion is the fundamental factor that determines the success of grafting (Pina and Errea 2005; Aloni *et al.* 2010). This involves the alignment of cell layers from which new cells proliferate from both rootstock and scion, producing a callus tissue that integrates within the spaces between the rootstock and scion (Aloni 2010; Cookson *et al.* 2013; Cookson *et al.* 2014). New cambial cells differentiate from the callus, forming a continuous cambial connection between rootstock and scion. Repair of xylem and phloem can initially connect the rootstock and scion vasculature. After, the newly formed cambial layer in the callus produces new xylem towards the center of the plant and new phloem towards the outside. This new, integrated vasculature arising from the cambium is what constitutes a successful graft.

1.3.2 Changes resulting from water and nutrient uptake capacity of rootstocks

One of the most prominent proposed mechanisms for rootstock effects on scion growth is that these effects result from differences in the ability of particular rootstocks to take up water and/or nutrients. With regards to water uptake this is often thought about simply by equating increases in root water uptake capacity with enhanced growth. Numerous studies have compared differences in root hydraulic conductivity per unit surface area, length, or biomass (Lpr) and/or whole root system hydraulic conductance (Lsystem) with differences in scion growth. Interpretations of effect of Lpr on scion growth need to be integrated with Lsystem which takes into account differences in whole root system surface area (i.e. biomass) (Figure 1.3).

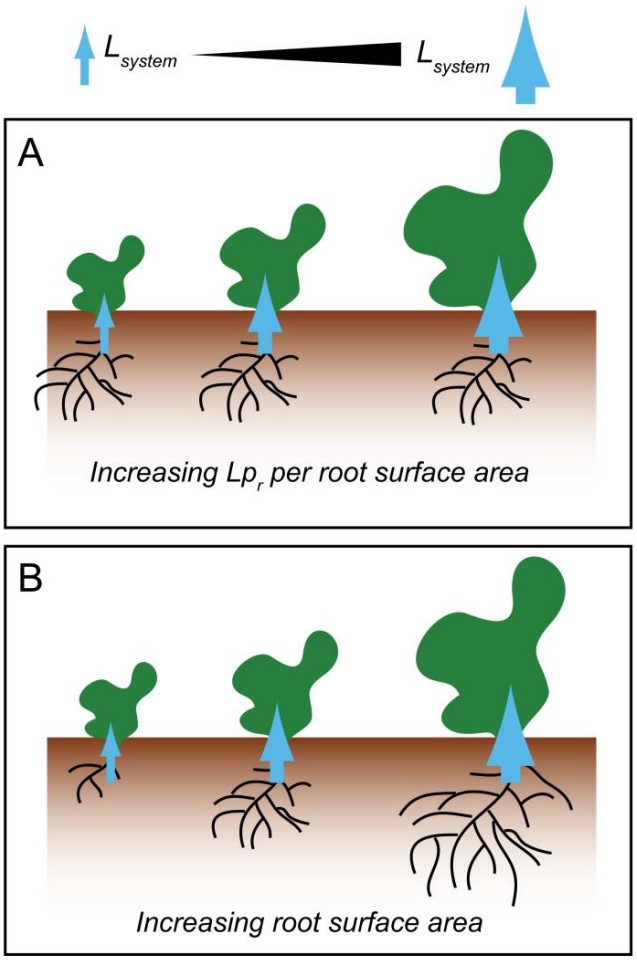

Figure 1.3 It has been hypothesized that increases in scion growth can be brought about by increases in the rootstock's ability to take up water. Both of the examples above show increases in whole root system hydraulic conductance (L_{system}) from left to right (blue arrows). However, increases in L_{system} can result from (A) increases in hydraulic conductivity per unit surface area (Lp_r), (B) increases in whole root system surface area, or a combination of both.

Many studies conclude that Lsystem is positively correlated with scion growth (Clearwater *et al.* 2004; Nardini *et al.* 2006; Solari *et al.* 2006; Lovisolo *et al.* 2007), while studies that have correlated Lpr with growth have more varied results. In grape, Gambetta *et al.* (2012) found that Lpr was positively correlated with scion growth and transpiration, and these results are similar to results in other species (Atkinson *et al.* 2003; McElrone *et al.* 2007). However, many studies in other species emphasize the importance of the size of the root system in conferring increased Lsystem in conjunction with increased vigor. Clearwater *et al.* (2004) found that Lp_r (normalized to leaf area) was greatest for the lowest vigor rootstocks and at the same time found that L_{system} was positively correlated with growth. The same results were found by Solari *et al.* (2006) in peach and Lovisolo *et al.* (2007) in olive. Alsina *et al.* (2011) suggested that seasonal changes in root proliferation may contribute to changes L_{system}.

Nutrient uptake is intimately connected with water uptake, but across species few studies have considered these two parameters together (Wright and Barton 1955; Russell and Shorrocks 1959; Cernusak *et al.* 2011; Kodur *et al.* 2010). In grape, rootstocks have been shown to affect the uptake of a variety of nutrients although the corresponding effect on scion growth is variable (Nikolaou *et al.* 2000; Kodur *et al.* 2010; Lecourt *et al.* 2015). For example, Nikolaou *et al.* (2000) correlated increased nitrogen and with increased shoot growth of Thompson Seedless grown on several rootstocks and Kodur *et al.* (2010) demonstrated that increased potassium uptake was correlated with the vigor of Shiraz across several rootstocks. In contrast, some works have demonstrated that although different rootstocks do lead to differences in nutrient accumulation in the scion, these differences are not associated with differences in growth and/or yield (Ruhl 1991; Dalbó *et al.* 2011). Viticulture could greatly benefit from more comprehensive studies integrating water and nutrient uptake with regard to rootstock effects on scion growth.

1.3.3 Rootstock-scion hormonal signaling

Rootstock induced changes in hormone levels, and/or signaling, are obvious mechanisms for rootstock effects on scion growth. Despite a wealth of literature on root-shoot hormonal signaling there is little work in the context of the grafted plant regardless of species (reviewed in Aloni *et al.* 2010). One of the most central hormone relationships controlling root and shoot growth is that of cytokinin and auxin especially given the long-distance transport of these hormones (reviewed in El-Showk *et al.* 2013). In peach, the ratio of cytokinin to auxin in the xylem sap of grafted scions was positively correlated with the rootstock vigor rating (Sorce *et al.* 2002). Rootstocks conferring higher vigor increased concentrations of cytokinin (specifically zeatin) in scion xylem sap. In grape, Skene and Antcliff (1972) correlated decreases in cytokinins with decreases in yield, and Nikolaou *et al.* (2000) correlated increases in cytokinin content with differences in the growth of Thompson Seedless grown on various rootstocks (420A, 110R, 99R, 41B, Kober 5BB, 8B Teleki, 1103P, 31R and 3309C) finding strong positive correlations with nitrogen content, and shoot growth rate (Figure 1.4).

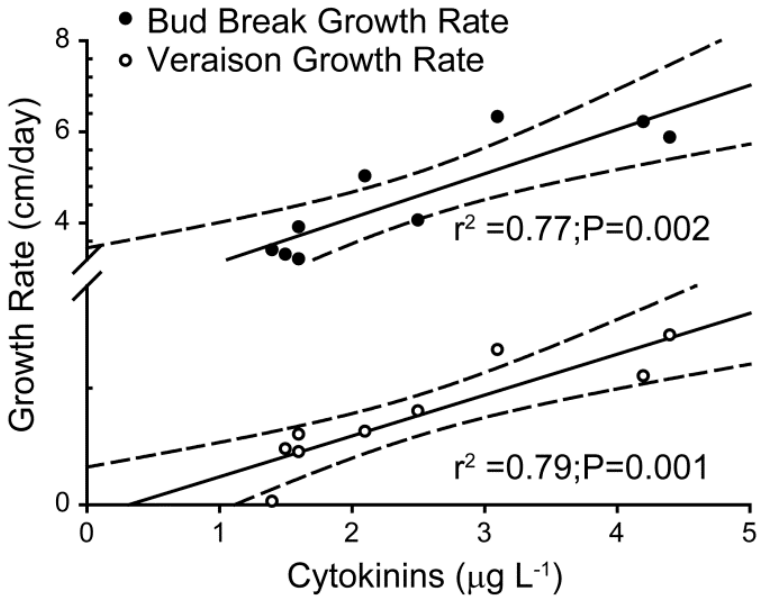

Figure 1.4 Regression analysis of data presented in Nikolaou *et al.* (2000). The authors presented data on differences in the growth patterns of Thompson seedless grown on various rootstocks (420A, 110R, 99R, 41B, Kober 5BB, 8B Teleki, 1103P, 31R and 3309C) and quantified concentrations of cytokinins in xylem sap. Cytokinin concentration was highly correlated with shoot growth rate both at bud break (closed circles) and veraison (open circles). R^2, P values, and 95 % confidence intervals are presented

Abscisic acid (ABA) is also a strong candidate for mediating rootstock effects on shoot physiology especially under drought (discussed below). Cotton rootstocks have been shown to modulate leaf senescence associated with changes in ABA and cytokinin levels (Dong *et al.* 2008). Dwarfing apple rootstocks that induced smaller sized trees were associated with elevated levels of ABA (Tworkoski and Fazio 2015). In grape, when Shiraz was grafted onto seven different rootstocks differences between stomatal behavior where strongly correlated with differences in ABA concentration in shoot xylem sap, and these differences were correlated with differences in vegetative and reproductive growth (i.e. pruning weight and yield) (Soar *et al.* 2006). One of the great challenges facing future studies aimed at understanding the role of hormones is discriminating between the direct effects of root derived hormones transported to the shoot from the indirect effects of changes in shoot hormone metabolism that result from rootstock effects on other parameters (e.g. root Lp, nutrient uptake, etc.).

1.3.4 Exchange of genetic information through grafting

In other plant species there is evidence that there is genetic exchange of DNA and/or RNA through the graft union although there is almost no information on grape specifically. Early work

in red pepper showed that hereditary changes of some inherited traits such as fruit color and fruit position can be induced by grafting (Ohta and Van Chuong 1975) and later Pandey (1976) suggested a theory of genetic hybridization resulting from grafting. More recently, work has demonstrated that grafting can result in gene transfer via either large DNA pieces or entire plastid/chloroplast genomes, but this phenomenon is restricted to the graft site (Stegemann and Bock 2009; Stegemann *et al.* 2012). Fuentes *et al.* (2014) established a fertile allopolyploid plant between two species via the method of grafting. Several studies have demonstrated the long-distance transport of microRNAs across graft unions in cherry, potato, and rapeseed (Buhtz *et al.* 2010; Bhogale *et al.* 2014; Zhao and Song 2014). In grape, messenger RNA molecules have been found to pass across the graft union and the authors provide evidence that the movement of these RNAs may be developmentally dependent (Yang *et al.* 2015). This is an important area of future study considering the possible application of transgenic rootstocks to deliver specific molecular regulators to a non-transgenic scion thereby avoiding the possibility of transgene transfer via the flowers.

1.4 Effects of rootstocks on scion response to drought

Cultivated grapevines can face undesirable growing conditions (e.g. drought, high salinity, nutrient deficiency, frost, etc.) that result in stress, among which drought has attracted much attention especially in the context of climate change. Water contributes to grapevine vegetative and reproductive growth and ultimately influences canopy size, yield, and berry composition (Keller 2015). Consequently, drought can have negative impacts on vine growth, yield, and possibly even grape and wine quality.

Grapevines adapt to drought through changes in both physiology and structure in order to maintain growth and development (reviewed in Lovisolo *et al.* 2010). The most well studied of these responses is stomatal closure, which reduces transcriptional water loss but also CO_2 availability in mesophyll and thus photosynthesis (e.g. Koundouras *et al.* 2008; Meggio *et al.* 2014). Grapevines are often characterized as being iso- or anisohydric based on their stomatal sensitivity to decreases in plant water status (e.g. Schultz 2003; Vandeleur *et al.* 2009; Rogiers *et al.* 2012) and there is evidence that these differences in behavior maybe under genetic control (Coupel-Ledru *et al.* 2014). At the same time some authors argue that these distinctions are artificial, providing examples of the same genotype exhibiting both behaviors depending on different growth conditions (Lovisolo *et al.* 2010; Chaves *et al.* 2010). Stomatal regulation's impact on water use efficiency and productivity is extremely complex. For example, water stress can negatively impact vine growth and yield but at the same time improve intrinsic water use efficiency (the ratio of net CO_2 assimilation to stomatal conductance) (Koundouras *et al.* 2008).

The root system is intimately connected with water availability in a drying soil. Rootstocks contribute to the control of scion transpiration under drought (Carbonneau 1985; Düring 1994; Iacono *et al.* 1998; Padgett-Johnson *et al.* 2000; Soar *et al.* 2006; Koundouras *et al.* 2008; Marguerit *et al.* 2012), and although the precise mechanisms are not completely understood, it is

thought to involve a combination of hydraulic and hormonal root-to-shoot signaling (Lovisolo 2010). Different rootstock genotypes differ in their drought resistance and studies have demonstrated a genetic component of rootstock control over scion transpiration and hormonal signaling forming the foundation for the breeding of drought resistant rootstocks (Soar *et al.* 2004; Marguerit *et al.* 2012; Rossdeutsch *et al.* 2016).

The studies examining rootstock effects on scion transpiration and water status under drought consistently demonstrate rootstock effects, but the effects are highly variable likely resulting from the intersection of rootstock/scion genotype and environment. For example, Koundouras *et al.* (2008) investigated leaf and whole-plant physiological and structural responses of Cabernet Sauvignon grafted onto 1103P and SO4 in field conditions. Under water stress, SO4 maintained higher stem water potential, net CO_2 assimilation rate, and leaf density however stomatal conductance, transpiration rate, and WUE were all unaffected by rootstock. These results are congruent with Düring (1994) where rootstocks improved leaf photosynthesis rate by increased carboxylation efficiency, while stomatal conductance was consistent among different rootstock-scion combinations. In contrast, other works demonstrate strong coordinated effects of rootstocks on both stomatal conductance and CO_2 assimilation (Iacono *et al.* 1998; Padgett-Johnson *et al.* 2000; Soar *et al.* 2006). Padgett-Johnson *et al.* (2000) observed coordinated rootstock effects on both stomatal conductance and CO_2 assimilation but without any difference in plant water status. Taken together these studies suggest that rootstocks can influence CO_2 assimilation through effecting photosynthetic machinery and leaf structure independently of stomatal conductance and/or via changes in stomatal conductance. It would be the hope that increased knowledge of the underlying mechanisms driving these nuanced responses could someday be leveraged in the development of new rootstocks.

1.5 Possible mechanisms involved in rootstock mediated drought resistance

Uncovering the details concerning the mechanisms behind rootstock effects on drought resistance is essential for improving vineyard management and guiding breeding efforts aimed at developing new drought resistant rootstocks. A large number of studies have investigated the mechanisms of stomatal control under drought. The roles of both direct hydraulic, stomatal closure resulting from decreases in leaf water potential and turgor, and hormonal control via ABA or other chemical signals, have been recognized for years (reviewed in Comstock 2002). McAdam and Brodribb (2015) recently demonstrated that direct hydraulic control predominates in basal plant lineages (e.g. ferns, gymnosperms) and that ABA control predominates in angiosperms. However, they did find some angiosperm species that exhibited a more mixed type of control. This suggests that there could be a various integrations of hydraulic/hormonal control across species.

Rootstocks have the ability to impact both hydraulic and hormonal signaling pathways (Figure 1.5). Root derived production and transport of ABA is a longstanding hypothesized mechanism for inducing stomatal closure but other root-derived chemical signaling pathways (e.g. pH) have

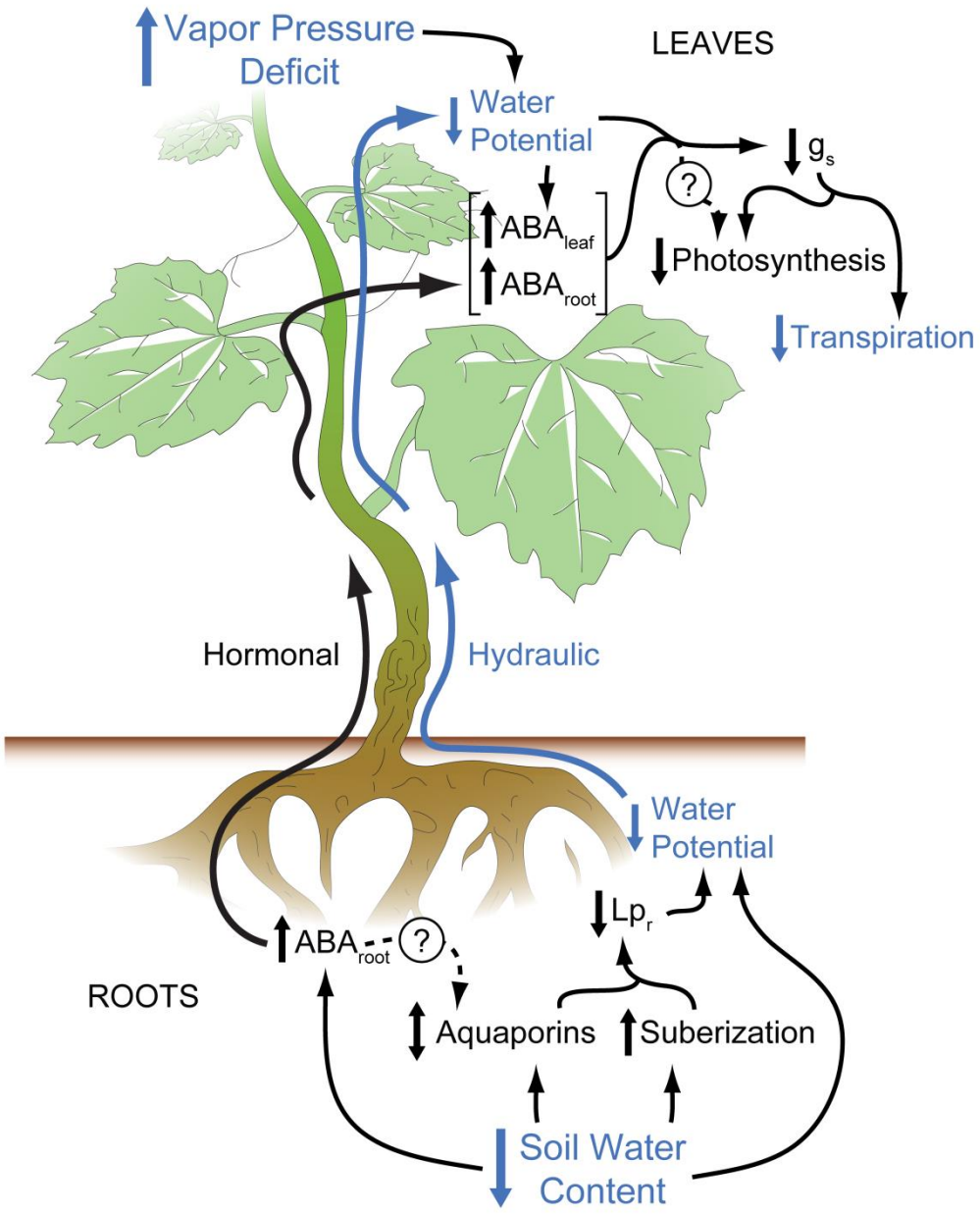

Figure 1.5 A simplified summary of the possible mechanisms through which rootstocks influence scion behavior under drought. The two most prominent hypothesized signaling pathways modulating stomatal conductance, transpiration, and photosynthesis are hormonal (black) and hydraulic (blue) signaling. Hormonal signaling results from the production and long distance transport of chemical signals (e.g., ABA) from the root to the leaves. Hydraulic signals likely involve decreases in root hydraulic conductivity (Lp_r) resulting in decreases in water potential that impact stomatal conductance. These root-derived signaling pathways are integrated with the same mechanisms originating in the leaves.

been proposed (reviewed in Davies *et al.* 2005; Jia and Zhang 2008). Additionally, hydraulic signals could induce leaf-derived ABA stomatal closure (Christmann *et al.* 2007; Dodd 2013; McAdam and Brodribb 2015). In a classic split root experiment in grape, Düring (1990) provided evidence for a root-derived chemical signal as stomatal closure occurred in the absence of declines in leaf water potential and turgor. In another split root experiment Lovisolo *et al.* (2002) also reported decreased stomatal conductance without changes in leaf or stem water potential but associated with increases in ABA. Other studies have found that rootstocks differentially affect decreases in stomatal conductance under drought and that these decreases are inversely correlated with increases in ABA concentrations in xylem sap (Soar *et al.* 2006) or leaves (Iacono *et al.* 1998).

Although hydraulic signaling is often discussed it is poorly studied in grape. Under well-watered conditions grapevine rootstocks differ in their root hydraulic conductivity/conductance (Lovisolo *et al.* 2008; Alsina *et al.* 2011; Gambetta *et al.* 2012; Tramontini *et al.* 2013) and drought stress leads to decreases in root hydraulic conductivity (Vandeleur *et al.* 2009; Barrios-Masias *et al.* 2015). Changes in the expression and activity of root aquaporins likely contribute to these decreases in conductivity under drought (Vandeleur *et al.* 2009). There are interesting connections between ABA, aquaporin activity, and root hydraulic conductance but their integration is poorly studied (discussed in Maurel *et al.* 2010). Changes in root hydraulic conductance can potentially influence leaf water potential (e.g. Else *et al.* 1995; Brodribb and Hill 2000). Thus, decreases in root hydraulic conductivity should lead to decreases in leaf water potential and turgor; a hydraulic signal. Tombesi *et al.* (2015) hypothesized that stomatal closure is mediated by direct hydraulic control but maintained by ABA suggesting that over longer time frames (i.e. weeks to months) root-derived ABA may be responsible for the maintenance of stomatal closure.

Drought stress leads to structural changes via the development of suberin lamellae in apoplast of particular root tissues that likely contribute to the sustained decrease in root hydraulic conductivity (Steudle 2000b). In grapevine, a suberized exodermis and endodermis can be found starting from the maturation zone in fine root tips (Figure 1) (Gambetta *et al.* 2013) and water stress increases suberization of the exodermis and/or endodermis is often observed (Vandeleur *et al.* 2009; Lovisolo 2010; Barrios-Masias *et al.* 2015). Barrios-Masias *et al.* (2015) observed that drought induced earlier and greater root suberization in the less drought-resistant genotype, 101-14MGt, than in 110R. These structural changes could potentially lead to a more enduring decrease in root hydraulic conductivity that could increase the sensitivity of plant water status to changes in the vapor pressure deficit (Maurel *et al.* 2010).

Long term water deficit can lead to other changes in structure that influence drought resistance such as changes in xylem vessel structure (Lovisolo and Schubert, 1998). These changes have the potential to alter important characteristics such as xylem resistance to embolism, but it not known to what extent rootstocks would potentially influence these longer term structural changes. This may be an interesting subject of future study.

1.6 Breeding drought resistant rootstocks

In the context of climate change there is an increasing focus on the development of new drought resistant rootstocks. The question of what specific qualities would constitute a drought resistant rootstock is a complicated one. Maintaining productivity and yields under stress is an obvious goal, but this must be balanced with the vine's ability to protect itself against long-term damage to its hydraulic function. It is interesting that those rootstocks that are traditionally categorized as high vigor also tend to be those categorized as drought resistant. This suggests that their drought resistance may result in part by having a more expansive root system resulting from increased vigor.

The control of stomatal conductance and other traits associated with drought resistance have genetic components (e.g. Juenger *et al.* 2005; Street *et al.* 2006; Marguerit *et al.* 2012; Coupel-Ledru *et al.* 2014). In grape, studies in non-grafted plants have revealed genetic differences in with regard to the transcriptional regulation of ABA metabolism and signaling (Rossdeutsch *et al.* 2016) and identified QTLs for behavior under drought for scion traits such as transpiration, leaf water status, and whole plant hydraulic conductance (Coupel-Ledru *et al.* 2014). However, to date there remains only one study aimed at discovering the genetic architecture responsible for rootstock effects on scion transpiration during drought. Marguerit *et al.* (2012) identified QTLs that were associated with differences in the decline of transpiration in response to decreasing soil water. Some of these QTLs co-localized with genes involved in the regulation of drought responses including numerous ABA biosynthesis and signaling components.

Understanding the genetics responsible for rootstock control over scion behavior is an incipient field of study. The complexity of the responses, putative mechanisms, and interactions with environment present significant challenges, but the breeding of drought resistant rootstocks is critical for the development of new sustainable approaches to address climate change in viticulture.

1.7 Conclusions

The mechanisms involved in the influence of grapevine rootstocks on scion growth and drought resistance and the interactions between rootstock and scion in a grafted system are far from being complete. Grafting is required in the cultivation of grapevine in most areas in the world, and rootstocks have a wide range of impacts on scion behavior (summarized from Keller 2015; Ollat *et al.* 2016). The study of rootstock-scion interaction is incredibly complex integrating structural changes at the graft interface, hydraulic integration, hormonal communication, and even exchange of genetic materials. Moreover, studies concerning the influences of rootstocks on scion growth, fruit composition, or wine quality do not always produce consistent results, possibly due to experimental conditions (e.g., potted vs field, young vines vs old vines), soil type and/or climatic conditions, scion variety, etc. Meta-analysis studies could be useful to better understand and integrate the studies that have already been carried out. Further studies aimed at understanding the physiology and genetics responsible for rootstock control over scion behavior

could benefit by combining different approaches (genetic, transcriptomic, metabolic, hydraulic, etc.) in the same study. There is still a lot to be gained from investigations in un-grafted material creating a foundation of understanding regarding the differences between the rootstock genotypes themselves.

Finally, grapes are of considerable economic importance. Future research bears a responsibility to move towards the application of new technologies in the vineyard. One of the most common goals posited by grapevine researchers is the development of new rootstock varieties that meet growers' demands, especially in the context of climate change. The complexity of the responses, putative mechanisms, and interactions with environment present significant challenges, but the breeding of drought resistant rootstocks is critical for the development of new sustainable approaches to address climate change in viticulture.

Acknowledgments

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Chapter 2 Literature review Part II

2.1 Introduction

This chapter is complementary to the published review in which topics regarding plant root growth and development, factors impact root growth (including water stress), root hydraulics and water relations as well as aquaporins are further discussed.

2.2 Root growth and development

Plant growth and development is a complex process and the dynamics of plant growth is the result of the interaction between the internal growth mechanisms and the external impacts of environmental conditions (Walter and Schurr 2005, Walter *et al.* 2009). Plant development is characterized by post-embryonic organogenesis mediated mostly by meristems, which allows plants, as sessile organisms, to grow continuously and indeterminately through their lifetime, to maximize the capture of resources and to respond appropriately to biotic and abiotic signals (Doerner *et al.* 1996, Hodge 2009, Baskin 2013, Gallagher 2013, Ramirez-Parra *et al.* 2017).

Roots are organs evolved with a functionally integrated vascular system and play multiple roles in water absorption, nutrient uptake, and anchorage (Pritchard 1994, Kenrick 2002). It is evident that optimized root growth and development can favor overall plant productivity, and thus is a highly desirable trait for manipulation in plants (Winicov 2000). Plant roots have been intensively studied as an ideal subject for investigating growth mechanisms due to the distinctive morphogenesis features of root apex: easily accessible, linear organization, radial symmetry, all developmental stages discernible at all times, relatively few differentiated cell types, clearly distinguishable zones along the length of the root indicating various developmental processes, and nearly indefinite growth (Schiefelbein and Benfey 1991, Baskin 2013).

As mentioned earlier, four distinct developmental zones can be designated along the length of a root tip where root growth is confined to: root cap, meristematic zone, elongation zone, and maturation zone (Figure 2.1, Taiz and Zeiger 2002). The differentiation of these four developmental zones is not absolute as there is considerable overlap in the cellular processes occurring in various zones (Schiefeibein and Benfey 1991, Taiz and Zeiger 2002). Root growth is the result of the linear arrangement of cell division, expansion growth and differentiation along the root tip (Walter *et al.* 2002). Embryogenesis initiated plant development and established primary meristems which constitute the primary root apical meristem (Taiz and Zeiger 2002, Casson and Lindsey 2003).

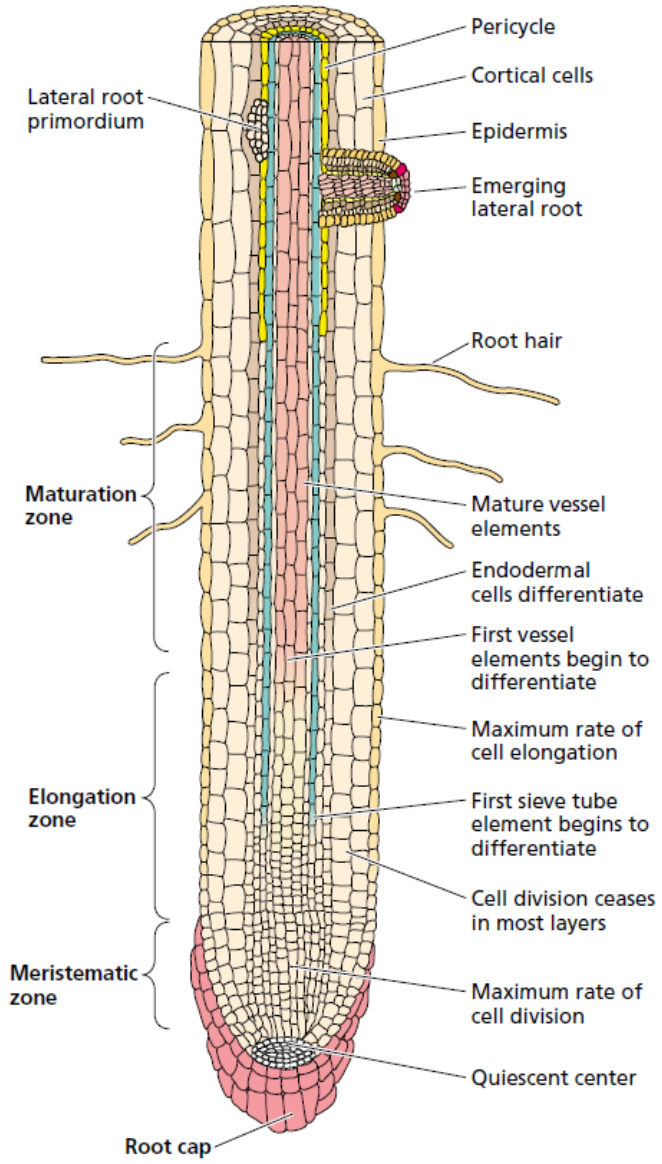

Figure 2.1 Simplified diagram of a primary root showing the root cap, the meristematic zone, the elongation zone, and the maturation zone (Taiz and Zeiger 2002).

The primary root apical meristem generates only the primary root which is often long-lived and may continue to grow through the life of the plant (Taiz and Zeiger 2002, Gallagher 2013). Unlike the primary root apical meristem, the lateral root meristem has its origin in post embryogenesis and is established from the cell divisions in the pricycle in mature and non-growing regions of the root (Taiz and Zeiger 2002, Casson and Lindsey 2003). Therefore, lateral roots emerge from the pericycle of the parent root post-embryonically. Figure 2.2 (Gambetta *et al.* 2013) displays an example of the anatomical structure of a grapevine fine root as well as patterns of suberization along the length of a fine root.

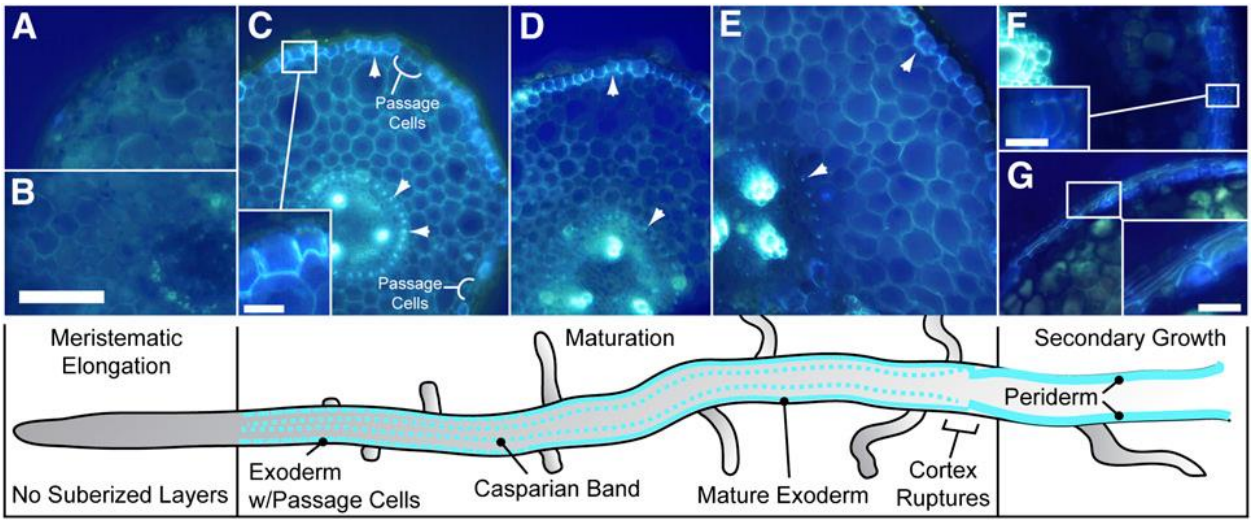

Figure 2.2 Patterns of suberization along the length of fine roots in grapevine rootstock variety 110R (*Vitis berlandieri* x *Vitis rupestris*). Light-blue staining indicates suberization. Bars = 100 mm (main panels) and 20 mm (insets) (Gambetta *et al.* 2013)

In most dicotyledons and gymnosperms, root growth consists of primary growth and secondary growth. Root primary growth results from the activity of root apical meristem in which cell division is followed by cell elongation. After elongation in a given region is complete, secondary growth may take place. Two lateral meristems are involved in secondary growth: the vascular cambium, which gives rise to the secondary xylem and secondary phloem; and the cork cambium, which produces the periderm that replaces the epidermis and constitutes the protective outer layer in woody roots (Taiz and Zeiger 2002). Figure 2.3 (Gambetta *et al.* 2013) illustrates the developmental anatomy of a grapevine fine roots consisting of both primary and secondary growth.

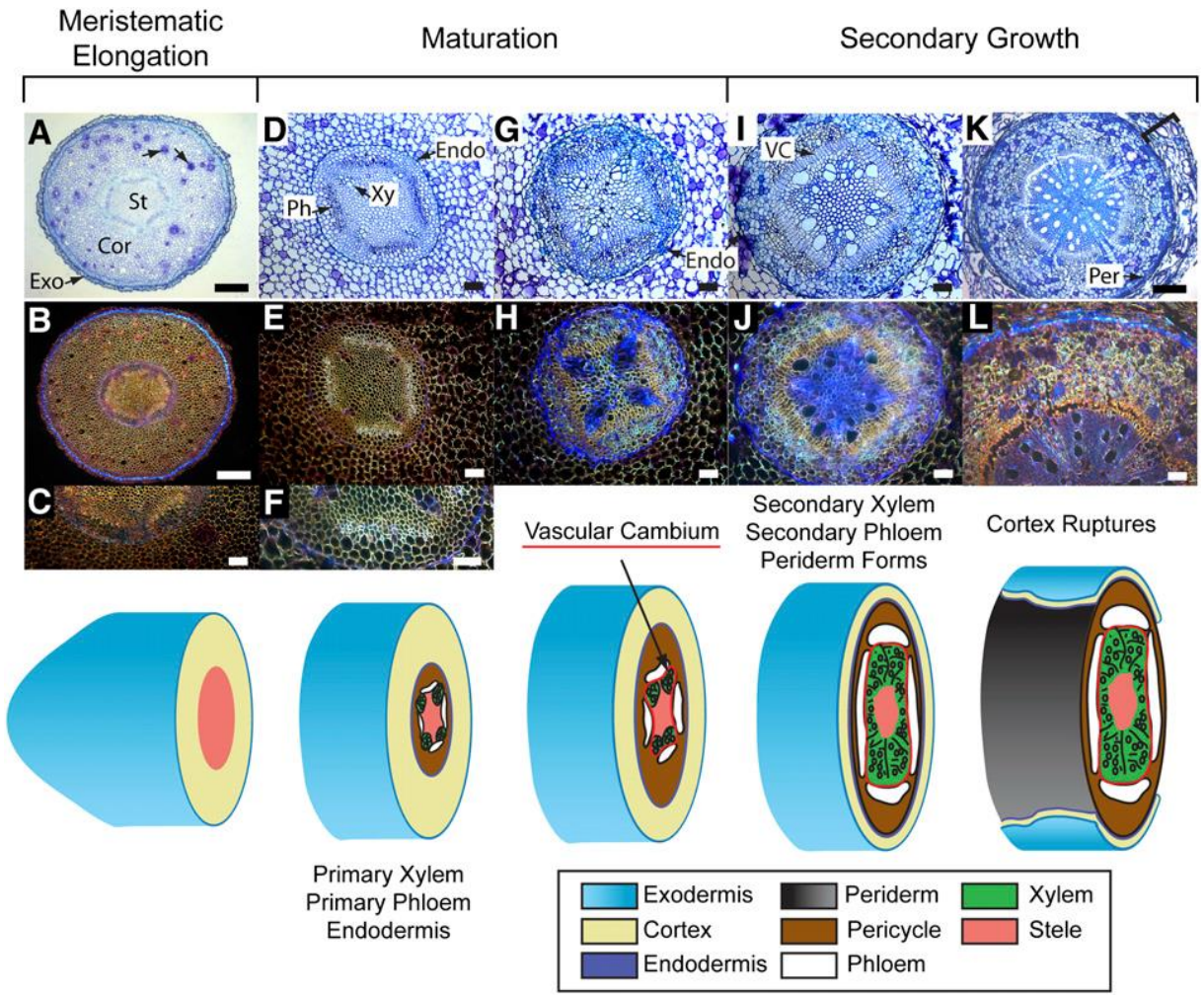

Figure 2.3 Primary and secondary growth of fine roots in grapevine rootstock variety 110R (*Vitis berlandieri* x *Vitis rupestris*). Exo = exodermis, Cor = cortex, St = stele, Xy = primary xylem, Ph = primary phloem, Endo = endodermis, VC = vascular cambium, Per = periderm. Bars = 200 mm (A, B, and K), 40 mm (F), and 80 mm (all others) (Gambetta *et al.* 2013)

2.3 Advances in the study of root growth and development

2.3.1 Measuring roots

The study of plant root growth and development is a challenging process and requires a great methodological and human effort largely due to its hidden nature (Nagel *et al.* 2009, de Herralde *et al.* 2010). Many techniques have been developed to increase the accessibility and visibility of plant roots. Investigation of the development of root systems seems to date back to the 18th century with the work of Duhamel du Monceau on the root systems of trees. The oldest method of examining root systems was to dig them out, which consumes a lot of energy and time, and in

addition, is destructive (Kramer and Boyer 1995). Nonetheless, if the primary goal of the study is to determine root biomass in the soil volume, direct excavation is still the most advised method (de Herralde *et al.* 2010). One form of excavation commonly employed in the study of root distribution is to create a trench and get rid of the soil, thus a profile wall is established for the observation (Kramer and Boyer 1995, de Herralde *et al.* 2010). Later, a soil-filled box with one glass wall to observe root growth was introduced by Sachs (1873), which gradually led to the development and application of non-invasive root observation chambers called rhizotrons to monitor a fraction of roots (Huck and Taylor 1982, Kramer and Boyer 1995, Dusschoten *et al.* 2016). Normally rhizotrons exist in two forms: underground chambers with transparent observing panel(s) built into soil, or big containers with transparent sides which are usually covered during the experiment (Huck and Taylor 1982, de Herralde *et al.* 2010). Information on root growth can be obtained by directly measurement on the transparent wall or by taking photographs during the growing process and then analysing the digital images with softwares such as ImageJ, RootEdge (Kaspar and Ewing 1997), RhizoScan, and WinRhizo (Nagel *et al.* 2009, de Herralde *et al.* 2010). Originally proposed by Bates (1937), minirhizotrons have also come into use (Taylor *et al.* 1990, Kramer and Boyer 1995). Briefly speaking, the minirhizotron system consists of installing clear plastic tubes about 5 cm in diameter and 2 or 3 m long in the soil at an angle about 30 to 45 degrees from the vertical, and afterwards, root growth can be recorded at the soil-tube interface by lowering down a camera and a fiber optic illumination device in to the tube anytime during the period of the experimentation (Taylor *et al.* 1990, Kramer and Boyer 1995). The recommended timing of installation of the tubes is a few days after the plants are planted (Taylor *et al.* 1990). In spite of the advantages of the rhizotron and minirhizotron system, such as it being non-destructive and allowing for extensive and successive measurements, they have disadvantages such as high cost, environmental modifications, and minimum number of tubes required (Huck and Taylor 1982, Taylor *et al.* 1990, and Nagel *et al.* 2009).

Hydroponic and solid cultures are also good options especially for the evaluation of important root traits of plants at an early growth stage and have been widely and frequently used (Jones 1982, Tuberosa *et al.* 2002, Nagel *et al.* 2009). For example, Koyama *et al.* (1995) designed a method to grow *A. thaliana* seedlings hydroponically and studied successfully the impact of aluminum ions on root elongation rate in *A. thaliana*. Price *et al.* (1997) evaluated a hydroponic screen system to determine root growth of 28 rice (*Oryza sativa* L.) varieties and furtherly identified and genetically analyzed varieties suitable for producing segregating populations. What is noteworthy is that Price *et al.* (1997) and Loresto *et al.* (1983) provided strong correlation between root growth in hydroponics and root growth in the field. Apart from hydroponics, solid media have been widely adopted as well to investigate root growth and other physiological behaviors under certain conditions. With agar medium, roots can grow on the surface by placing it vertically, which allows marking and imaging the roots on the surface of a growth matrix (Weele *et al.* 2000). As early as 1941, Day grew excised tomato roots in agar medium and explored the effect of pyridoxine on their growth. Buer *et al.* (2000) investigated the root-wave phenomenon in Arabidopsis growing on medium culture and concluded that Arabidopsis root

growth in medium culture was responsive to the microenvironment of the Petri dish and to the chemical and structural properties of the growth medium. Medium culture was also used to study root growth responses to water deficit (Weele *et al.* 2000), and to nutrient deficiencies (Gruber *et al.* 2013), etc.

Image acquisition and analysis provide a powerful solution to examine the dynamics of the structure and development of root systems (French *et al.* 2012). French *et al.* (2009, 2012) and Fiorani (2013) summarized and discussed available software packages for root sequences. Some software packages are designated for certain cultivation systems, for example, RootTrace is designed to process images of roots grown on agarose plates (French *et al.* 2009).

When it comes to phenotyping the important characteristics of the root system architecture, the methods mentioned above all have indisputable limitations: direct excavation is destructive, rhizotrons and minirhizotrons cannot provide a full picture of the root system, hydroponics and medium culture provide an artificial growth environment (Dusschoten *et al.* 2016). The application of computed tomography (X-ray, γ-ray, and neutron), dual-energy scanning, nuclear magnetic resonance (NMR), as well as magnetic resonance imaging (MRI) has made it possible to acquire high resolution images of roots in natural soil (Asseng *et al.* 2000, French *et al.* 2012, Dusschoten *et al.* 2016).

2.3.2 Molecular analysis of root development

In addition to phenotypic analyses of plant root systems, new technologies in microscopy, molecular biology, and genetics have made it possible to improve our understanding of the mechanisms that control root development on a molecular level, which involves isolation and characterization of genes expressed in roots as well as tissue- and zone-specific gene expression in roots (Schiefeibein and Benfey 1991, Rost and Bryant 1996). Changes in gene expression patterns in roots in response to external stimuli have been reported too. In the latest version of annotation of the *Arabidopsis thaliana* reference genome which covered more than 99% of all genes, it is reported that among the 27 596 protein-coding genes detected in 11 tissues, root and the reproductive tissues have the highest number of expressed genes with 19 414 and 19 380 genes, respectively (Cheng *et al.* 2017). Meanwhile, they also have higher fractions of tissue-specific genes, e.g., 285 root-specific genes. Moreover, the expression of many non-coding RNAs detected (4 560 in total) tends to exhibit a tissue-specific pattern, which was predominantly observed in reproductive tissues and root. A significant number of root developmental mutants have been identified and isolated in the model plant Arabidopsis as well as other species, which comprise two fundamental types: one is morphological mutants which show abnormal developmental patterns, and the other one is mutants that are not able to respond in a normal manner to external stimuli (Schiefelbein *et al.* 1993). The study of these mutants allows the discovery of responsible genes engaged in corresponding processes (Montiel *et al.* 2004). Moreover, some of these genes can encode transcription factors (TFs) which are master-control proteins that interact with cis-regulatory DNA elements in the promoter regions of target

genes or other transcription regulators and are capable of activating or repressing the transcription of multiple target genes (Czechowski *et al.* 2004, Montiel *et al.* 2004). As a result, transcription factors can regulate many biological processes as well as plant responses to external signals by temporarily and spatially controlling the transcription of their target genes (Montiel *et al.* 2004, Jin *et al.* 2013).

Birnbaum *et al.* (2003) elaborated a global gene expression map of the Arabidopsis root with microarray technology which localized the expression of over 22 000 genes within 15 various zones and allowed the correspondence between gene activity and cell fate as well as tissue specialization. Five separate GFP maker lines expressing green fluorescent protein (GFP) in stele, endodermis, endodermis plus cortex, epidermal atrichoblast cells, and lateral root cap, respectively, were used to obtain the gene expression data, and three developmental stages were profiled along the length of the root tip. Later on, to better elucidate all transcriptional patterns that occur in the root, Brady *et al.* (2007) generated a comprehensive high-resolution microarray expression map presenting almost all cell type-specific (14 non-overlapping cell types out of 15) spatiotemporal transcriptional profiles in a single Arabidopsis root. Developmental stages along the longitudinal axis of the root were profiled by microdissecting a single Arabidopsis root into 13 portions of approximately 3 to 5 cells within each portion. Temporal expression variation was assessed by analysing a second individual root.

Kohler *et al.* (2003) generated and analysed more than 7 000 expressed sequence tags (ESTs) from roots of poplar (*Populus trichocarpa* x *P. deltoides*) including almost 5 000 transcripts that are uniquely expressed in roots in order to better guide gene discovery in poplar root as well as to highlight genes involved in water and nutrient absorption and transport. Poroyko *et al.* (2005) used Serial Analysis of Gene Expression (SAGE) to examine the transcript abundances in maize root and produced a total of 161 320 tags resulting in the detection of at least 14 850 genes. Together with a set of virtual tags extracted from maize EST, this analysis contributed to the annotation of maize root transcriptome as well as to the commencement of relating maize root transcripts to functional groups and bio-chemical pathways (Poroyko *et al.* 2005).

Recent development in next-generation sequencing (NGS) technologies and assembly algorithms has made effective and comprehensive transcriptome sequencing possible (Cao and Zeng 2017). RNA-seq technology has been readily available to facilitate the determination of transcriptome complexity and the understanding of genetic regulation networks (Song 2016). De novo transcriptome sequencing has been performed in many plant species in addition to the model plant species. For example, Mitsui *et al.* (2015) investigated the transcriptome sequencing of another root crop radish (*Raphanus sativus* L.) during root development and identified a total of 54 357 genes. Furthermore, the authors analysed genes related to carbohydrate metabolism and pungency synthesis, which can be informative for breeding new cultivars with favorable traits. Li DM *et al.* (2015) performed an extensive transcriptome sequencing analysis in *Paphiopedilum concolor* root and detected 64 304 unigenes which were further functionally annotated and classified into putative functional categories. The authors also expressed their interest in

understanding *Paphiopedilum* root growth and development and therefore identified relative genes.

2.3.3 Transcription factors

In Arabidopsis, initially, about 1 500 transcription factors encoded by approximately 2 000 TF genes making up for around 5% of the genome were identified (Riechmann *et al.*, 2000, Hong 2015), among which 577 were detected in root and 331 were differentially regulated in different zones (Birnbaum *et al.* 2003). Czechowski *et al.* (2004) identified 35 putative root-specific expressed transcription factor genes out of 1 247 TF genes analysed in Arabidopsis. Therefore, it can be speculated that the expression of transcription factors accounts for a critical aspect in the sophisticated functional network of transcriptional regulation in root growth and development (Montiel *et al.* 2004, Gruber *et al.* 2009). A more recent transcription factor analyse has revealed a putatively complete set of 2 304 transcription factors in Arabidopsis and the information is also available for another 4 plant species with their genome sequences released (Riaño-Pachón *et al.* 2007). Montiel *et al.* (2004) reviewed the complex transcriptional regulation network connected by transcription factors in root development and characterized the transcription factors involved in the establishment and maintenance in primary root meristem, in root hair formation as well as in lateral root formation.

2.4 Factors influencing root growth

Theoretically, the mechanics of root growth include activities of cell division in root apical meristem just behind the tip, and cell expansion in elongation zone behind the meristem (Clark *et al.* 2003). It is generally accepted that the turgor pressure created when water moves across membrane and enters into the cell down a water potential gradient is the driving force for cell elongation which results in the growth of root (Pritchard *et al.* 2000, Clark *et al.* 2003, Wiegers *et al.* 2009). Notably water influx into cells is accompanied by nutrients uptake.

Plant root growth and development is controlled and influenced by intrinsic genetics and is also highly responsive to external environmental stimuli. Internal and external factors exert impacts on root growth include but are not restricted to: phytohormones, transcription factors, age, water availability, nutrients, soil properties, light (radiation), temperature, air humidity, and so on.

2.4.1 Light

It is suggested that root growth is tightly associated with carbon acquisition in roots which is predominately fueled by import from shoot through phloem. Therefore, despite growing underground and in darkness, light conditions at shoot can have a big impact on root growth (Walter and Nagel 2006). An early study from Eliasson (1978) investigated the impact of light on root growth and formation in fast growing *Pisum sativum* seedlings and reported that increased intensity of light favored the formation of roots. To further understand the role of photosynthetic products in light effect, exogenous sucrose at different concentration was added to the culture

solution. Root formation (number of roots) was significantly increased by the addition of sugar at low light intensity (8 W/m^2) while the effect of sucrose on root formation was smaller at high light intensity (40 W/m^2), and the effect was negative at the highest concentration. However, emergence of roots was delayed with the presence of sucrose at both high and low irradiances, and thus a decrease in root length was observed. Noland *et al.* (1997) conducted an experiment on jack pine seedlings planted in peat and vermiculite soil at different light intensities and observed that low light intensity significantly reduced photosynthetic rate, number and length of new roots as well as total nonstructural carbohydrate in roots. In this experiment, new root initiation and growth was well correlated with root starch depletion. Therefore, the authors assumed that new root growth was maintained at the cost of current photosynthate. As a matter of fact, the effect of light on root growth is complex. Lambers and Posthumus (1980) studied root growth of *Plantago lanceolata* L. and *Zea mays* L. cv. Campo under different light and humidity regimes in culture solution. Even though the rate of dry matter accumulation in roots decreased in *Plantago lanceolata* L. at low light intensity, the content of carbohydrates in roots and root total respiration were not affected by light intensity. Therefore, the authors came to the conclusion that photosynthesis was not a major factor in the regulation of root growth. Experiment made on *Zea mays* L. under different humidities implied that in spite of the decreased rate of dry matter accumulation in roots of *Zea mays* L. under low light intensity, this effect was not imposed via transpiration.

Given the inconsistent arguments in terms of the mechanisms involved in the effect of light intensity on root growth, Aguirrezabal *et al.* (1994) designed an experiment to study the relationship between root growth rate and intercepted photosynthetic photon flux density (PPFD) in sunflower (*Helianthus annuus* L.) in both field and growth chamber conditions. The results obtained in this study confirmed that the relationship between light intensity and root elongation is connected by carbon allocation. Additionally, the authors pointed out that this regulation process over root growth is relatively slow due to the transport of carbon resource through phloem. A more recent study undertaken by Nagel *et al.* (2006) in tobacco (*Nicotiana tabacum* L.) seedlings cultivated in agar culture medium again provided evidence that root growth is more pronounced under high light intensity. Further experimentation with external application of sucrose on isolated roots excised from shoots and transgenic tobacco with reduced sucrose synthesis ability uncovered the role of sucrose as signaling substance in this regulation process.

Another aspect worthy discussing concerning the influence of light on root growth is when roots are subjected to light and consequently light might turn into a stress factor. Even through unusual, roots may be exposed to light under certain situations such as penetration of sunlight several centimeters below the soil surface (Mo *et al.* 2015, Qu *et al.* 2017), small cracks on the surface of the soil which allow light to penetrate, unexpected abrupt temperature changes, earthquake, sever storm, etc (Yokawa *et al.* 2014). Besides, conversely, light is essential for emerging radicals to increase root growth rate shortly after seed germination on the ground (Yokawa *et al.* 2014).

Inhibitory effects of white light on the elongation of root have been reported (e.g., Torrey 1952, Pilet and Went 1956). Pilet and Ney (1978) managed to apply white light locally to either root cap or the elongation zone and found that root elongation rate of maize (*Zea mays* L.) was strongly and rapidly inhibited only when the part of root cap was illuminated.

Robert *et al.* (1975) reported that in cress seedlings (*Lepidium sativum* cv. curled green) exposed to continuous light production of ethylene was greatly increased while root length was strongly inhibited. Given the role of ethylene as a growth inhibitor, it can be assumed that light inhibits root growth by promoting the production of ethylene. To further understand the mechanisms of light-induced inhibition of root growth as well as the role of ethylene in the inhibition process, Eliasson and Bollmark (1988) compared root elongation rate and ethylene production of light-grown and dark-grown pea seedlings (*Pisum sativum* L. cv. Weibull's Marma). Furthermore, endogenous synthesis of ethylene was deliberately stimulated by the addition of 1-aminocyclopropane-1-carboxylic acid (ACC) to the growth solution under both growing conditions, and ethylene synthesis inhibitors aminooxyacetic acid (AAA), Co^{2+} or Ag^{+} was added with the intention to counteract light-induced root growth inhibition. The authors confirmed that ethylene was at least partly the inhibition factor resulted from root exposure to light as to some extent the down-regulation of root growth by light was counteracted by ethylene synthesis inhibitors.

Thanks to recent advances in molecular biology and biological technologies, more studies have pursued the mechanisms involved in root responses to light and offered more insights to our understanding of the mechanisms. Briefly speaking, plants exhibit phototropism, a behavior through which plant organs can respond to changes in light direction to optimize their growth and performance (Moni *et al.* 2014, Lee *et al.* 2017). The aboveground shoots show positive phototropism and grow toward to incoming light, while the underground roots show negative phototropism and tend to bend away from the light source (Yokawa *et al.* 2014). Plants are capable of responding rapidly and properly to light of different spectra due to their remarkably sophisticated and extremely sensitive light-sensing systems (Mo *et al.* 2015). Plants perceive and absorb light by protein molecules known as photoreceptors (Möglich *et al.* 2010) and furtherly transduce the perception of light into cellular and hormonal responses (Yokawa *et al.* 2013). Plants have six classes of photoreceptors including lightoxygen-voltage (LOV) sensors, xanthopsins, phytochromes, blue-light sensors using flavin adenine dinucleotide (BLUF), cryptochromes, and rhodopsins, which permit them to sense light at wavelengths from the spectral UV-B to FR regions (Möglich *et al.* 2010, Briggs and Lin 2012, Mo *et al.* 2015). Notably, most of the photoreceptors are also present in roots, even when they are growing in the dark, including phytochromes, cryptochromes, phototropins, and ultraviolet receptors (Briggs and Lin 2012, Yowaka *et al.* 2014, Mo *et al.* 2015, Lee *et al.* 2016). Numerous studies have revealed evidence proving that roots can respond to light and result in significant morphological and developmental changes, such as primary root growth, lateral root initiation, negative phototropism, gravitropism, root nodule formation, nitrate uptake, tuberization, and greening (Mo

et al. 2015, Lee at al. 2017). Therefore, it is obvious that roots can perceive light signals directly or receive long-distance transduced signals from the aboveground tissues and consequently provoke photomorphogenic responses even though they grow in the dark under natural conditions (Lee *et al.* 2017, Qu *et al.* 2017). Moreover, light-related pathways can be tightly connected with phytohormonal signaling and regulate plant growth and development simultaneously (Yokawa *et al.* 2014).

2.4.2 Temperature

Temperature is one of the principle environmental elements plants are exposed to through their entire life journey and temperature fluctuation can affect greatly the growth and activities of root systems (Faget *et al.* 2013). Root systems face both diurnal fluctuations and annual variations regarding temperature changes.

Temperature is a major player in affecting the growth and development of root tissues. Even through uninterrupted growth may be maintained in evergreen tree species, forest observations from MacDougal (1930) showed that growth ceased in Monterey pine tree when cambium temperature lowered to 8 °C and suggested that the minimum of 8 °C may be considered as the lower limit of temperature of the cambium under which accretions to the trunk may take place. A two-year observation obtained from filed observation frames in *Pinus echinata* Mill. (shortleaf pine) and *Pinus. taeda* L. (loblolly pine) by Turner (1936) provided evidence of correlation of number of active roots and daily average growth with temperature. During the two growing seasons, seasonal periodicity of root growth with fast growing period with a large number of active roots and slow growing period with a small number of growing roots were recorded, which, according to the author, were corresponded with higher temperature and considerable rainfall and lower temperature or less rainfall, respectively. In winter when mean temperature was low (around 11 °C), high rainfall was not able to accelerate root growth.

In grapevines, it has been shown that optimum root growth occurs at around 30°C (Richards 1983). However, this value may change depending on the genotype (Clarke *et al.* 2015). In general, grapevine roots exhibit a very distinct growth pattern observed in all different rootstocks (Delrot *et al.* 2001). Two predominant growing phases at flowering and harvest were found respectively, which possibly resulted from the temperature requirement and the sink demand related to the growth of shoots and roots (Van Zyl 1988, Delrot *et al.* 2001,). Bud growth is promoted in early spring when soil temperature is lower than air temperature. Root growth is apparently delayed until the emerging of leaves who serve as active sinks for assimilates. Later on, berry growth will be competing with root growth from fruitset until harvest (Delrot *et al.* 2001). In terms of the effects of soil temperature on grapevine growth, Woodham and Alexander (1966) have reported that bud-break and shoot development, shoot and root growth, and percent fruit-set were considerably higher at high root temperature (30 °C) than at low root temperature (11 °C) in Thompson Seedless grapevines.

As the metabolic and catabolic processes in plants are temperature-sensitive, according to Clarke *et al.* (2015), the reason why roots grow faster in warmer soil could be resulted from a greater rate of root carbohydrate reserve catabolism at higher temperature, which will provide more energy and C skeleton to stimulate root growth. Rogiers *et al.* (2013) have confirmed that elevated root-zone temperature at 22.6 °C~24.8 °C from budburst to fruitset in Shiraz (*Vitis vinifera* L.) stimulated the mobilization of carbohydrate reserve in roots as well as the translocation of nitrogen and potassium to berry and petiole, and accelerated shoot growth and reproductive development (e.g., flowering, fruitset, and véraison). In a similar experiment conducted by Clarke *et al.* (2015), stimulated root growth, root branching, mobilization of carbohydrate reserve in roots and canopy development have also been reported. Moreover, warmer soils have also enhanced the uptake of primary nutrients (e.g., N, P, K, Ca and B) by increased number and length of active roots. Experiment with ^{15}N isotope labelling provided evidence that higher N content in grapevines exposed to warmer soil temperature was resulted from improved nutrient absorption. However, notably, the concentration of other macronutrients in petioles was either lower (Mg and Na) or unchanged (Fe and Zn) from vines growing in warmer soils, which probably indicated a complex process of nutrient uptake or suggested that the optimal temperature for each element absorbed by roots is not the same.

2.4.3 Soil properties

The ability of roots to grow and to explore soil for water and nutrients is an important element determining the performance of plant growth (Clark *et al.* 2003). Soil properties can be divided into physical properties, chemical properties, and biological properties, and they can all limit root growth in soil (Bengough *et al.* 2011). In terms of physical limitations to root growth, mechanical impedance, soil water content, and aeration are major players to slow down the growth and development of root systems (Bengough *et al.* 2005, Bengough *et al.* 2011). Many studies have demonstrated that in general mechanical impedance decreases root elongation rate and increases root radial expansion (Sarquis *et al.* 1992). Tardieu (1988) reported that root density and water absorption decreased in maize growing in wheel compacted soil. Sarquis *et al.* (1992) revealed that a mechanical pressure of 100 kPa on the soil increased ethylene production by four-fold and root diameter by seven-fold, but decreased root elongation by 75%. Valentine *et al.* (2012) concluded that root elongation rate is severely limited. Hosseini *et al.* (2017) showed that an increase in root medium penetration resistance from 1.17 to 5.96 MP led to increased root diameter and decreased root volume in wheat (*Triticum aestivum* L. cv. Chamran) seedlings.

2.4.4 Water availability

Changes in root growth under water deficit depend largely on the degree of the stress level. When plants face water deficit, the development of the root system is less inhibited than shoot growth, and may even be stimulated, ending up with increased root biomass and deeper rooting depth (Sharp and Davis 1989). It has been reported that shoot growth can be limited even prior to the development of reduced water potentials in the aerial parts of the plant (Saab and Sharp 1989,

Gowing *et al*. 1990). Maintenance of root growth at water potentials that are low enough to inhibit shoot growth is obviously advantageous to sustain an adequate water supply to the whole plant (Sharp *et al*. 2004). Meanwhile, reduced above-ground growth will decrease transpiration as well as demand for water supply (Hoogenboom *et al*. 1987), which provides another protective mechanism.

Rodrigues et al. (1995) have reported a stimulation of root growth in an herbaceous plant lupin (*Lupinus albus* L.) after 15 days of water stress treatment during flowering. Pre-dawn leaf water potentials of well-watered seedlings were maintained at around -0.1 MPa while those were dropped to below -0.6 MPa in water-stressed seedlings. At the end of the treatment, water shortage induced a significant increase in fine root length per unit soil volume and a slight increase in fine root dry weight. The increase in fine root dry weight was more pronounced in deeper soil layers. Sustained root growth under drought is likely an adaptive response resulting from osmotic adjustment or an enhanced cell wall loosening capacity (Saab *et al*. 1992, Hsiao and Xu 2000, Chaves *et al*. 2002, Sharp *et al*. 2004). Besides, the plant hormone abscisic acid (ABA) has been proposed to be an important factor in the regulation of root growth and play an essential role in plant differential growth responses to water deficit (Saugy *et al*. 1989, Saab *et al*. 1990). Barcia *et al*. (2014) investigated the consequences of water deficit in wheat (*Triticum aestivum* L. cv 75 Aniversario) root growth under a range of water potentials (from -0.03 MPa to -1.2 MPa). Apparently, root elongation rate was reduced under water stress in correlation with the magnitude of the stress.

2.5 Root hydraulic conductivity

Root hydraulic conductivity indicates the ability of roots taking up water. Hydraulic conductivity and changes in response to internal and external stimuli are highly variable, partially due to the relative contributions of different components of water transport (Steudle 2000a). A composite transport model has been suggested to explain the various pathways of water entering roots based on their complex anatomical structure (Steudle and Peterson 1998, Steudle 2000a). The three different pathways described by Steudle and Peterson (1998) are: 1) the apoplastic path around protoplasts which can be affected by changes of the anatomical structure, e.g. suberization of exodermis and endodermis (Barrios-Masias *et al.* 2015), 2) the symplastic path through plesmodesmata, and 3) the transcellular path across cell membranes, which can be largely adjusted by aquaporins (water channel proteins).

Root hydraulic conductivity may change due to root development and aging (Steudle 2000a). Melchior and Steudle (1993) studied the changes in radial hydraulic conductivity during root development in onion (*Allium cepa* L.) and discovered that root hydraulic conductivity was smaller and more variable in more basal zones of the root due to more developed exodermal Casparian bands and/or suberin lamellae in the endodermis or exodermis. Root hydraulic conductivity may also vary in response to external stimuli such as water stress. Generally, decreased root hydraulic conductivity is observed when plants are exposed to drought constraints

as demonstrated by numerous studies across various species. Among perennial plants, Rieger (1995) reported reductions in root hydraulic conductivity to varying degrees in peach (*Prunus persica* L. Batsch), olive (*Olea europaea* L.), citrumelo (*Poncirus trifoliata* Raf x *Citrus paradisi* Macf.) and pistachio (*Pistachia integerrima* L.), and Trifilo *et al.* (2004) reported decreased root hydraulic conductivity in ailanthus (*Ailanthus altissima*). Down-regulations of root hydraulic conductivity under water stress have also been observed in annual herbaceous plant species such as common bean (*Phaseolus vulgaris*, Aroca *et al.* 2006), lettuce (*Lactuca* sativa, Aroca *et al.* 2008), and rice (*Oryza sativa* L., Gao *et al.* 2010). A large amount of studies has been carried out in desert plant species such as *Agave deserti* (North and Nobel 1998, 2000, 2004), *Opuntia ficus-indica* L. (North and Nobel 1992, 1996), *Ferocactus acanthodes* (North and Nobel 1992), *Opuntia acanthocarpa* (Martre *et al.* 2001). In grapevine, Vandeleur (2007) observed significant decreases in whole root system hydraulic conductivity under drought in two scion varieties Chardonnay and Grenache and one rootstock variety 101-14. Barrios-Masias *et al.* (2015) evaluated fine root hydraulic conductivity under different moisture conditions and across different rootstock varieties and found that root hydraulic conductivity decreased for both 101-14 and 110R under dry conditions with a hydrostatic driving force. However, with an osmotic driving force, reductions in root hydraulic conductivity under dry conditions were only observed in 101-14. Although decreases in root water uptake and root hydraulic conductivity are generally observed in roots exposed to drought, increases in root hydraulic conductivity have been observed under certain specific circumstances. For example, Siemens and Zwiazek (2004) reported an up-regulation in root hydraulic conductivity in solution culture-grown aspen (*Populus tremuloides*) seedlings subjected to mild water stress by being exposed to a sealed high humidity chamber for 17 hours. However, conversely, root hydraulic conductivity was reduced in roots under severe water stress. The initial decrease of hydraulic conductivity upon roots exposure to drought constraints is suggested to be a protective mechanism to prevent water from leaking back to soil which has a decreasing water potential and lower than that of the roots (Vandeleur 2007, Aroca *et al.* 2011).

2.6 Aquaporins

2.6.1 Introduction on aquaporins

Aquaporins are channel-forming transmembrane proteins present in plasma and intracellular membranes in all eukaryotes and most prokaryotes (Chaumont *et al.* 2001). Initially, aquaporins' water transport capabilities were discovered and functionally characterized in human red blood cells (Benga *et al.* 1986, Denker *et al.* 1988, Preston and Agre 1992) and later in plants (*Arabidopsis thaliana*) with the functional characterization of a vacuolar water-transporting protein, γ-TIP (Maurel *et al.* 1993). After the discovery of plant aquaporins, many studies have been conducted in order to elucidate their structure, function, and regulation across numerous plant species (reviewed in Tyerman *et al.* 2002, Maurel *et al.* 2008, Chaumont and Tyerman 2014). Aquaporins were first characterized as water channels, but they are also recognized to

contribute to the transport of other small neutral molecules (e.g., glycerol, urea, boric acid, silicic acid), gases (e.g., CO_2, ammonia) and even ions under certain circumstances (Tyerman *et al.* 2002, Sakurai *et al.* 2005, Maurel *et al.* 2008, Maurel *et al.* 2015).

Aquaporins fall within an ancient superfamily of membrane proteins called major intrinsic proteins (MIPs). The MIP family consists of a large number of homologs, and can be subdivided into four major subfamilies based on sequence similarity, which may also indicate their sub-cellular localizations (Johanson *et al.* 2001, Alexandersson *et al.* 2005). The plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), and the nodulin26-like intrinsic proteins (NIPs), comprise the major subfamilies (Maurel *et al.* 2008, Chaumont and Tyerman 2014, Li *et al.* 2014). These three groups of aquaporins have been intensively studied and well-documented. The small basic intrinsic proteins (SIPs) include only a few isoforms localized in the ER (e.g., 3 homologs in Arabidopsis) (Ishikawa *et al.* 2005, Maurel *et al.* 2015). In addition to these four well-conserved subfamilies present in all plant species, several additional novel types of aquaporins have been distinguished but with a less ubiquitous presence among plant species. For example, the uncategorized X intrinsic proteins (XIPs) were recently discovered, but are absent in some higher plants. The GlpF-like intrinsic proteins (GIPs) and the hybrid intrinsic proteins (HIPs) were discovered in moss and algae, but are absent in vascular plants (Li *et al.* 2014, Maurel *et al.* 2015).

The structure of the MIP gene family, like many plant gene families, has resulted from numerous gene duplications resulting in groups of closely related isogenes (e.g., Johanson *et al.* 2001, Cannon *et al.* 2004). In general, many of these closely related isogenes have overlapping patterns of expression, but some have undergone sub-functionalization with regard to their specific developmental and/or tissue related expression patterns (Adams and Wendel 1999). This is certainly the case for MIP family members where many isogenes display tissue and/or developmentally specific expression patterns. Tissue specific expression of MIP isogenes has been observed in numerous species including corn (Chaumont *et al.* 2001, Gaspar *et al.* 2003, Opitz *et al.* 2016), rice (Sakurai *et al.* 2005, Sakurai *et al.* 2008), Arabidopsis (Weig *et al.* 1997), and ice plant (Yamada *et al.* 1995) among other species. On an even finer scale specific isogenes have been associated with specific cell types within organs (e.g., Kirch *et al.* 2000, Heinen *et al.* 2009). Most previous studies were not comprehensive across all MIP family members or across organs/tissues.

Grapevine is a plant species of economic and cultural importance and one of the first to have its genome sequenced (Jaillon *et al.* 2007). This information allowed for the characterization of large gene families such as the MIP family, and indeed this genome information was immediately utilized by Shelden *et al.* (2009) to integrate cDNA and genome information in characterizing the MIP family members in grapevine. Since then the original Pinot noir genome has been greatly improved and there has been a wealth of microarray and RNA-seq studies examining a huge breadth of circumstances in grapevine. Furthermore, new tools and approaches have been developed for analyzing the nature of genome duplications (Wang *et al.* 2012), as well as gene

expression and cis-regulatory element structure (e.g., Wong *et al.* 2017). These improvements allow for a more comprehensive analysis of the grapevine MIP gene family.

2.6.2 Regulation of aquaporin activity

Reversible phosphorylation is a potential posttranslational mechanism of plant aquaporin regulation. Normally plant aquaporins can be phosphorylated at a serine (Ser) residue localized on its N-terminal or C-terminal tail (Chaumont *et al.* 2005). An early study on α-TIP in bean (*Phaseolus vulgaris* L.) (*Pv*TIP3-1) seeds has discovered that this protein was phosphorylated at a single Ser residue near the N-terminal tail by a calcium dependent protein kinase (Johnson and Chrispeels 1992). Later on, regulation of aquaporin activity by phosphorylation was demonstrated by Maurel *et al.* (1995) by showing that phosphorylation of an α-TIP expressed in *Xenopus* oocytes increased their osmotic water permeability. Direct evidence of phosphorylation of Ser residues in the N-terminal and C-terminal tails of several plant aquaporins has been reported. In vitro labeling has proved that a a spinach (*Spinacia oleracea*) leaf plasma membrane aquaorin PM28A (*So*PIP2-1) was phosphorylated at the Ser-274 lacolized at the C-terminal tail in a Ca^{2+}-dependent manner by a plasma membrane-associated protein kinase (Johansson *et al.* 1996), while in vivo labeling of the same aquaporin demonstrated that the amino acid Ser-274 was phosphorylated in response to increasing apoplastic water potential and dephosphorylated in response to decreasing water potential (Johansson *et al.* 1998). Guenther *et al.* (2003) showed that the phosphorylation of soybean nodulin 26 (*Gm*NOD26) on Ser 262 stimulated its water permeability and was catalyzed by a symbiosome membrane-associated calcium-dependent protein kinase. Furthermore, phosphorylation was increased in vivo by osmotic stresses (water deprivation and salinity) (Guenther *et al.* 2003). Daniels and Yeager (2005) first demonstrated the phosphorylation of *Phaseolus vulgaris Pv*TIP3-1 with mass spectrometry analyses in vitro. With X ray diffraction, Törnroth-Horsefield *et al.* (2006) investigated the structural mechanism of aquaporin phosphorylation in *So*PIP2-1 and observed that two highly conserved serine residues, Ser 115 and Ser 274 of *So*PIP2-1, were dephosphorylated in response to water stress, which would cause loop D, typically longer for the PIP subfamily members, to block the pore of the aquaporin.

Plant developmental stages may mediate the regulation of aquaporin phosphosrylation (Chaumont *et al.* 2005). In bean seeds, phosphorylation of aquaporin *Pv*TIP3-1 reached a peak in developing seeds while decreased during seed imbibition (Johnson and Chrispeels 1992). Phosphorylation of soybean *Gm*NOD26 in symbiosomes peaked when nodules were mature and fully developed (Guenther *et al.* 2003).

In addition to phosphorylation, aquaporin methylation has also proved to be a possible mechanism for posttranslational aquaporin regulation. *At*PIP2-1 was detected to be methylated at two adjacent residues, Lys3 and Asp6, on its cytosolic NH_2-terminal tail (Santoni *et al.* 2006). Although methylation of *At*PIP2-1 did not alter the intrinsic water permeability of the aquaprorin, it could be involved in aquaporin subcellular trafficking (Santoni *et al.* 2006, Maurel *et al.* 2015).

The pH- and pCa-dependent gating is another possible mechanism involved in the regulation of plant PIPs. The inhition of PIP water transport by H^+ is primarily due to the protonation of a highly conserved His residue of loop D (Maurel *et al.* 2015). Tournaire-Roux *et al.* (2003) uncovered the molecular mechanism for cytosol acidosis related inhibition of water uptake on both whole-root and cell bases and reported that His197 localized in loop D is the primary residue responsible for pH-mediated gating in *At*PIP2-2. When His197 is substituted by an alanine residue the impact of cytosol acidosis is reduced. On a structural level, in *So*PIP2-1, His193 is positively charged at acidic pH and interacts with other amino acid residues to stabilize loop D in a closed pore conformation (Törnroth-Horsefield *et al.* 2006, Frick and Järvå 2013, Maurel *et al.* 2015). The presence of divalent cations may also lead to the reduction of membrane water permeability. Gerbeau *et al.* (2002) observed that in the presence of Mg^{2+} and Ca^{2+} the hydraulic conductivity of intact Arabidopsis cells was decreased by 35% and 69%, respectively. Structurally, similar to H^+-mediated aquaporin gating, divalent cation can directly bind between the NH_2 terminus (Gly30 and Asp31) and loop D through loop B to stabilize the closed pore conformation, thereby water transport of PIPs will be inhibited (Maurel *et al.* 2015).

Regulation of plant aquaporins may also occur by heteromerization between different isoforms. Aquaporins are generally found to be tetrameric (Chaumont *et al.* 2005). Due to their highly conserved structure, members of a same plant aquaporin subfamily may physically assemble as heterotetramers, thereby enabling multiple molecular and functional combinations (Maurel *et al.* 2015). The expression of maize (*Zea mays*) *Zm*PIP1-2 or *Zm*PIP1-1 in *Xenopus* oocytes does not increase the osmotic water permeability coefficient (P_f), whereas coexpression of *Zm*PIP1-2 with *Zm*PIP1-1, *Zm*PIP2-1, *Zm*PIP2-4, or *Zm*PIP2-5 increased the P_f of oocytes (Fetter *et al.* 2004). Fetter *et al.* (2004) demonstrated the physical interaction and heteromerization between two *Zm*PIP1s as well as between *Zm*PIP1-2 and *Zm*PIP2 isoforms.

2.6.3 Regulation of aquaporins in response to water stress

In the case of water stress, no consistent trend has been found concerning changes of aquaporin gene expression level as there is evidence for down-regulated, up-regulated, and unchanged expression of different aquaporin genes. For example, in common bean (*Phaseolus vulgaris* L. cv. Borlotto), after 4 days withholding water, transcript abundance of *Pv*PIP1-3 and *Pv*PIP2-1 was up-regulated, while the expression of *Pv*PIP1-2 and *Pv*PIP1-1 was drastically down-regulated and remained unchanged, respectively (Aroca *et al.* 2007). In maize (*Zea mays* L. cv. Potro), after 4 days without watering, the expression of *Zm*PIP1-1 gene was up-regulated, the expression of *Zm*PIP2-5 and 2-6 genes were down-regulated, and the expression of *Zm*PIP1-2, 1-5, 2-1 and 2-2 genes maintained constant (Ruiz-Lozano *et al.* 2009). In tobacco (*Nicotiana tabacum* cv. Samsun), drought stress significantly down-regulated the transcript abundance of *Nt*PIP1;1 and *Nt*PIP2;1 genes while up-regulated the transcript abundance of *Nt*AQP1 gene (Mahdieh *et al.* 2008). In two grapevine scion varieties, the expression of *Vv*PIP2-2 gene was not modified under water stress in both varieties, while the expression of *Vv*PIP1-1 gene was up-regulated in Chardonnay but remained unchanged in Grenache (Vandeleur *et al.* 2009).

Based on the expression data, it is difficult to clarify the function of aquaporins in response to water stress as well as in regulating root water uptake. However, each PIP gene could play a specific role under specific circumstances (Aroca *et al.* 2011), and some studies have provided evidence to support this point. For example, overexpression of Arabidopsis PIP aquaporin gene *At*PIP1b in transgenic tobacco plant significantly increased plant growth rate and vigour, transpiration rate, as well as photosynthetic efficiency under favourable growth conditions but not under drought or salt stress conditions (Aharon *et al.* 2003). Similarly, under favourable growing conditions, overexpressing tobacco PIP aquaporin gene *Nt*AQP1 in Arabidopsis and tomato plants increased shoot growth, transpiration rate and photosynthetic efficiency (Sade *et al.* 2010). Conversely, antisense suppression of *Nt*AQP1 gene resulted in decreased root hydraulic conductivity and reduced water stress resistance but showed negligible modification in transpiration rate (Siefritz *et al.* 2002). Overexpression of a wheat PIP2 aquaporin gene TaAQP7 increased drought tolerance in tobacco plants (Zhou *et al.* 2012), and likewise overexpression of tomato PIP genes *Sl*PIP2-1, *Sl*PIP2-7 and *Sl*PIP2-5 enhanced drought tolerance in tomato and Arabidopsis plants (Li R *et al.* 2016). In grapevine 'Brachetto', by overexpressing *Vv*PIP2-4N gene (the most expressed PIP2 gene in root in Brachetto) in transgenic grape plants, Perrone *et al.* (2012) have concluded that *Vv*PIP2-4N had a substantial function in the regulation of root water relations under well-watered conditions but not under water-stressed conditions. Moreover, the authors suggested that other signals induced by water stress such as ABA might override the role of aquaporins and cause the lack of aquaporin-mediated regulation under water stress.

Chapter 3 Root growth and influence of water stress

3.1 Introduction

Plant roots are essential organs where water and nutrient uptake takes place and where particular stress signals from the soil (e.g., water deficit, salinity) are perceived and transduced to other parts of the plant. Root development can be strongly affected by growing conditions. The ongoing climate change is causing increased temperature and anomalous precipitation, and as a result, drought has been an environmental constraint facing many areas. Effect of drought on plant development and growth depends largely on the degree of the stress level. Under moderate level of water stress, stimulated root growth has been observed (Rodrigues *et al*. 1995), while under high level of water stress root growth can be inhibited, and Barcia *et al*. (2014) has reported a correlation between the reduction of root elongation rate and the level of water stress. Moreover, when plants face water constraints, in general, the development of root system is less inhibited than shoot growth, and may even be stimulated, ending up with increased root biomass and deeper rooting depth (Sharp and Davis 1989). It has been reported that shoot growth can be limited even prior to the development of reduced water potentials in the aerial parts of the plant (Saab and Sharp 1989, Gowing *et al.* 1990).

The aim of the experiments in this chapter was to first develop a sand-based rhizotron system to study root growth, and then furtherly to evaluate individual and average root growth rate under well-watered and water-stressed conditions between two contrasting *Vitis* rootstock genotypes. Moreover, fluctuations of root growth rate in response to temperature as well as root anatomical changes in response to water deficit were assessed.

3.2 Materials and methods

3.2.1 Plant materials and growing conditions

Two commonly used grapevine rootstocks, RGM (Riparia Gloire de Montpellier, *Vitis riparia*) and 110R (110 Richter, *Vitis berlanderi* x *Vitis rupestris*), were studied in this project, as RGM is considered as low drought resistant and low vigor, while 110R is considered as highly drought resistant and medium viogr. One-year old dormant grapevine cuttings were purchased from the vine nursery and were stored in a cold chamber (4 °C) until the time of utilization, and before plantation, a rehydration process of at least 24 hours in water at 25 °C is necessary. After being rehydrated, grapevines were planted in cylinder rhizotrons (height 40 cm x diameter 14 cm) with 100 % sand and only one bud at the top node was kept for shoot growth. The rhizotrons were placed in a greenhouse without any lighting, temperature or humidity control. The plants were watered until filed capacity right after plantation and were then subjected to an automatic irrigation system with standard nutrient solution. The composition of the nutrient solution was:

2.5 mM KNO_3, 0.25 mM $MgSO_4 \bullet 7H_2O$, 0.62 mM NH_4NO_3, 1 mM $NH_4H_2PO_4$, 9.1 mM $MnCl_2 \bullet 4H_2O$, 46.3 mMH_3BO_3, 2.4 mM $ZnSO_4 \bullet H_2O$, 0.5 mM $CuSO_4$ and 0.013 mM $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ (Tandonnet *et al.* 2010). Iron was supplied as 8.5 mg/L Sequestrène 138 (EDDHA 5.9% Fe) and the final pH of the nutrient solutino was 6.0 (Tandonnet *et al.* 2010). After an establishment period (usually around three weeks after the plantation), for each genotype, plants were randomly assigned to two water treatments: well-watered condition and water-stressed condition. Plants under well-watered conditions were irrigated as during the establishment period and were referred to as control (CT), and plants under water-stressed conditions did not received any water supply during the period of treatment and were referred to as water-stressed (WS).

In order to record root growth rate, a piece of transparent plastic film was pasted around each rhizotron. Root growth rate was obtained by daily marking the position of root tips on the wall of rhizotrons when the root apices were visible. In addition, a piece of thick and non-transparent lightproof paper was wrapped around each rhizotron in order to prevent the exposure of roots to light. Normally white fine roots with light yellow tips can be observed through rhizotrons in two weeks after the plantation. Figure 3.1 shows the rhizotron system and how root length was measured. Root growth was measured daily for the entire duration of the experiment in all the experiments conducted. Fresh weight (g) of whole root system and leaves were recorded for RGM in the July-August 2016 experiment and for 110R in the August-September 2016 experiment.

In the growing season of 2015, three independent experiments were conducted in May, July-August, and September-November for both genotypes and were considered as early, mid, and late growing season, respectively. In the growing season of 2016, similarly, three independent experiments were carried out in May-June, July-August, and August-September. However, all the 110R plants in the July-August experiment did not survive due to unsuccessful rooting of the cuttings, so only RGM was available in the July-August experiment. Then more 110R plants were planted later which were utilized in the August-September experiment. Table 3.1 displays a summary of the plant composition for each experiment.

In the growing season of 2015, midday stem water potential (Ψ_{midday}) was measured as an indicator for plant water status at the end of the experiment just prior to sampling in the first experiment, and all subsequent experiment meausred pre-dawn leaf water potential ($\Psi_{predawn}$). Samples of individual roots with known growth rate from the first and the third experiments were collected and used to study the relative expression of seven aquaporin genes (*Vv*PIPs), and root tips from the second experiment were sampled to observe the differences in anatomical structure of well-watered and water-stressed plants.

In the growing season of 2016, $\Psi_{predawn}$ was always measured in order to monitor the stress level of the plants. In the May-June experiment, samples of individual roots with known growth rate were collected for the measurement of root hydraulic conductivity (Lp_r). In the July-August

experiment (only RGM), samples of individual roots with known growth rate were harvested for the study of aquaporin gene expression. In the August-September experiment (only 110R), samples of individual roots with known growth rate were collected for both Lp_r measurement and the study of aquaporin gene expression.

In this chapter, all the growth data from all the experiments were reported.

Table 3.1 Plant composition of the six experiments conducted

	2015 growing season						2016 growing season			
Month	**May**		**July-August**		**September-November**		**May-June**		**July-August**	**August-September**
Genotype	RGM	110R	RGM	110R	RGM	110R	RGM	110R	RGM	110R
N° of plants CT	3	3	4	3	3	4	11	9	15	9
N° of plants WS	3	3	3	3	3	3	14	11	21	10

3.2.2 Measurement of water potential

Pre-dawn leaf water potential ($\Psi_{predawn}$) and/or mid-day stem water potential (Ψ_{midday}) were measured with a pressure chamber to monitor the water status of the plants. One leaf from the middle part of the stem was sampled to determine $\Psi_{predawn}$ before sunrise with the help of a magnifying glass and a torch light. For Ψ_{midday}, a leaf (also from the middle part of the stem) was placed in a plastic bag wrapped with aluminum foil paper for one hour prior to the measurement between 12h00 and 14h00.

3.2.3 Epifluorescence microscopy

Fresh roots with known growth rate were sampled and kept in 70% ethanol at 4°C for further observations of their anatomical structure. A berberine-aniline blue fluorescent staining method was used to stain root sections (Brundrett *et al.* 1998). Root cross-sections were taken at 5 different locations along the root, categorizing sections into 5 groups based on their distances from the root tip: 0-1 cm, 1-2 cm, 2-4 cm, 4-7 cm, and 7-8 cm. Root segments from each group were fixed in 6 % low gelling temperature agarose and cut into 50 µm thick pieces with a vibrant Microtome with razor blade (Microm 650V). After the staining procedure, root sections were mounted on a slide and observed with an epifluorescence microscope Zeiss Axiophot equipped with an Amira software.

Figure 3.1 Rhizotron system used in the experiments. Roots can be observed through the transparent walls of the cylinder rhizotron. A sheet of transparent film was attached around the cylinder and roots were followed every day with hand drawing. A piece of thick and non-transparent lightproof paper was wrapped around each rhizotron in order to prevent the exposure of roots to light.

3.2.4 Statistical analysis

Treatment effect on plant water potential was evaluated using a one-way analysis of variance (one-way ANOVA, $p < 0.05$, Tukey's HSD test). Treatment effect on average root growth rate of each day within one genotype and genotype effect on average root growth rate of each day under the same water condition were analyzed using student's t- test ($p < 0.05$). All ANOVA and t-test were run in R version 3.3.1 (2016-06-21) (R Core Team) and all graphs were made with SigmaPlot (Version 11.0, Systat Software).

3.3 Results

3.3.1 Individual and average root growth rate

Root growth of two grapevine rootstock varieties, 110R and RGM, was observed in two growing seasons, the 2015 and 2016 growing season. Individual root growth is very heterogeneous, although drought treatment reduces root elongation on average, individual root growth rate still varies enormously. Individual root growth rate can fall in a wide range, which, in our experiment, is from less than 1 mm/day until more than 20 mm/day (maximum daily root elongation rate recorded: 25 mm/day, RGM, July 2016). Among the six independent experiments conducted, in most cases, around 93% to 99% of the single roots observed grew between 0 and 10 mm/day (A and B in Figure 3.2, 3.4, 3.5, and A in Figure 3.7), except for the growing period of July-August in 2015 and 2016, during which the ratio of roots elongating at less than 10 mm/day was 88% and 80%, respectively (A and B in Figure 3.3, and B in Figure 3.6). Under well-watered conditions, the peak of the individual root growth rate distribution curve occured at 3-5 mm/day (see A and B in figure series from Figure 3.2 to 3.5, and A in Figure 3.6 and 3.7).

For both genotypes, average root growth rate fluctuated to a great extent during each growing period. A fast-growing phase was observed at the beginning of each growing period, and then roots tended to grow at decreased speed with time going on and with increased developmental changes (C and D in figure series from Figure 3.2 to 3.5, and B in Figure 3.6). At the late stage of a growing season (e.g., October-November 2015, C and D in Figure 3.4), average root elongation rate was maintained at a much lower level and showed more stability (approximately 2-3 mm/day from mid-October to mid-November, and approximately 1-2 mm/day in mid-November).

Changes in average daily root growth rate in function to plant developmental stage were explored as well (Figure 3.8). Average daily root growth rate is the mean of all root growth rates measured for one day for each condition. Four conditions are categorized in our study: RGM CT, RGM WS, 110R CT, and 110R WS. Plant developmental stage is expressed as day of experiment, with day 1 of experiment representing the first day on which root growth rate was recorded. All daily average root growth rates across six experiments conducted during two growing seasons for both RGM and 110R under both well-watered and water-stressed conditions were taken into account. Average daily root growth rate is very plastic, and the scattered points are very noisy (grey circles in Figure 3.8). However, for each day of experiment, when we grouped the average daily growth rates based on treatment and calculated the mean of the average daily root growth rate, we observed a clear trend of decrease for both well-watered (black line in Figure 3.8) and water-stressed (red line in Figure 3.8) roots with longer day of experiment. On average, roots under well-watered conditions showed higher growth rates compared with those under water-stressed conditions (Figure 3.8 lines).

3.3.2 Influence of water stress on root growth

Mid-day stem water potential and/or pre-dawn leaf water potential were measured to determine the water status of the plants. As expected, plant water potentials significantly decreased with prolonged drought treatment; compared with plants under water stress, well-watered plants maintained their water status at a high level. In the May 2015 experiment, drought treatment was performed during a period of 9 days until Ψ_{midday} dropped to around -1.0 MPa, while Ψ_{midday} of well-watered plants was maintained at around -0.4 MPa (panel A and B in Figure 3.2). In the July-August 2015 experiment, roots were sampled when $\Psi_{predawn}$ of water-stressed plants dropped to around -1.2 MPa while $\Psi_{predawn}$ of well-watered plants was always around -0.1 MPa (A and B in Figure 3.3). However, in the September-November 2015 experiment, plant water status was not significantly influenced by the stress treatment due to the weather conditions in the late growing season of the year (insets in A and B in Figure 3.4). In the three experiments conducted in the growing seasion of 2016, two water stress levels, low (WS-Low) and high (WS-High), were categorized by sampling plants at different time points during the period of water stress treatment. Normally, $\Psi_{predawn}$ of WS-Low dropped to around -0.3 MPa to -0.5 MPa (A and B in Figure 3.5, A in Figure 3.6 and 3.7), and $\Psi_{predawn}$ of WS-High dropped to around -1.5 MPa to -1.8 MPa (A and B in Figure 3.5, A in Figure 3.7) or to around -1.0 MPa (A in Figure 3.6), while $\Psi_{predawn}$ of well-watered plants was always maintained at around -0.1 MPa (A and B in Figure 3.5, A in Figure 3.6 and 3.7). Genotype did not have an impact on changes of plant water potentials during the process of continuous lack of watering in our experiment.

For both RGM and 110R, water stress treatment significantly reduced root elongation rate, with the exception of the experiment carried out in September-November 2015, where stopping irrigation did not change plant water status (panel A and B Figure 3.4) due to the weather conditions in the late stage of the growing season. In May 2015, under water deficit, the distribution of single root growth rate shifted to a lower rate, and the average root growth rate was significantly decreased. For RGM, the peak of the root growth rate distribution curve moved slightly from 3-4 mm/day to 2-3 mm/day, and for 110R, the peak shifted from 4-5 mm/day to 2-3 mm/day (A and B in Figure 3.2). With sufficient water supply, average root growth was maintained at around 4 mm/day for both cultivars at the end of the experiment period, while the growth rate reached to around 1 mm/day under drought treatment (C and D in Figure 3.2).

In the other experiments, different levels of water stress were achieved during the drying down process, so two groups of stress levels were categorized as low and high level of water stress (WS-Low and WS-High). As mentioned above, root growth rate declined with prolonged drought treatment; however, changes in root growth under water stress depended also on the severity of the stress level. In the experiment from May-June 2016, for both RGM and 110R, individual root growth distribution curve under low level of water stress overlapped with the one under well-watered conditions, and the peak of the curve WS-Low stayed at 3-4 mm/day, which was the same as CT (A and B in Figure 3.5). In the experiment from July-August 2016 (only RGM) and August-September 2016 (only 110R), individual root growth rate distribution curve shifted

slightly to the lower rate, with the peak of curve moved from 4-5 mm/day to 3-4 mm/day, and from 4-5 mm/day to 2-3 mm/day, respectively for RGM and 110R (A in Figure 3.6 and 3.7). To the contrary, in the experiment from July-August 2015, individual root growth distribution curve switched slightly to higher rate under low level of water stress, with the peak of the curve moved from 3-4 mm/day to 4-5 mm/day for both varieties (A and B in Figure 3.3). In contrast, root growth distribution curve shifted strongly to lower speed under high level of water stress with the peaks of the curves falling at 1-2 mm/day (A in Figure 3.3, 3.6, and 3.7) or 2-3 mm/day (B in Figure 3.3, A and B in Figure 3.5).

Stress treatment also decreased root elongation rate on average for both genotypes (panel C and D in Figure 3.2, 3.3, 3.5, panel B in Figure 3.6 and 3.7). To be more specific, in May 2015, for RGM, average root growth rate was significantly reduced after 6 days without water (C in Figure 3.2); for 110R, average root growth was already affected after 2 days of stress treatment (D in Figure 3.2). In July-August 2015, for both cultivars, low level of water stress did not impose an impact to average root growth rate. For RGM, average root growth rate only dropped significantly at the end of the stress treatment (C in Figure 3.3), which was under high level of water stress when $\Psi_{predawn}$ dropped to around -1 MPa (inset in A in Figure 3.3). However, for 110R, even with $\Psi_{predawn}$ around -1 MPa (inset in B in Figure 3.3) under water-stressed conditions, average root growth rate showed a declined trend, but it was not statistically significant (D in Figure 3.3). As shown in panel C and D in Figure 3.4, average root elongation rate was not affected due to an inefficient stress treatment in the late growing season in 2015. In May-June 2016, unlike in May 2015, the average root growth rate of RGM was slightly increased under low level of water stress, and decreased under high level of stress, but this decrease was not statistically significant (C in Figure 3.5); the average root growth of 110R was not changed under low level of water stress and was significantly reduced under high level of stress (D in Figure 3.5). In July-August 2016, as in 2015, the average root growth rate of RGM was drastically affected in the last few days of the stress treatment (B in Figure 3.6). In the August-September experiment, the average root growth rate of 110R showed a slight decrease from the beginning of the stress treatment and fell significantly with continuous drought treatment (B in Figure 3.7).

3.3.3 Influence of genotype on root growth

Differences in root growth were observed between the two genotypes studied. In the early (e.g., May-June) and late (e.g., October-November) growing periods of a growing season, under well-watered conditions, 110R grew at a higher speed than RGM (E in Figure 3.2, 3.4, and 3.5); however, the differences disappeared under water deficit and the roots from both genotypes grew at a similar speed (F in Figure 3.2 and 3.5). In the middle period of a growing season (e.g., July-August), even under well-watered conditions, 110R lost its advantage in root growth and showed no difference with RGM (E in Figure 3.3); under drought, both genotypes again grew at a similar speed (F in Figure 3.3).

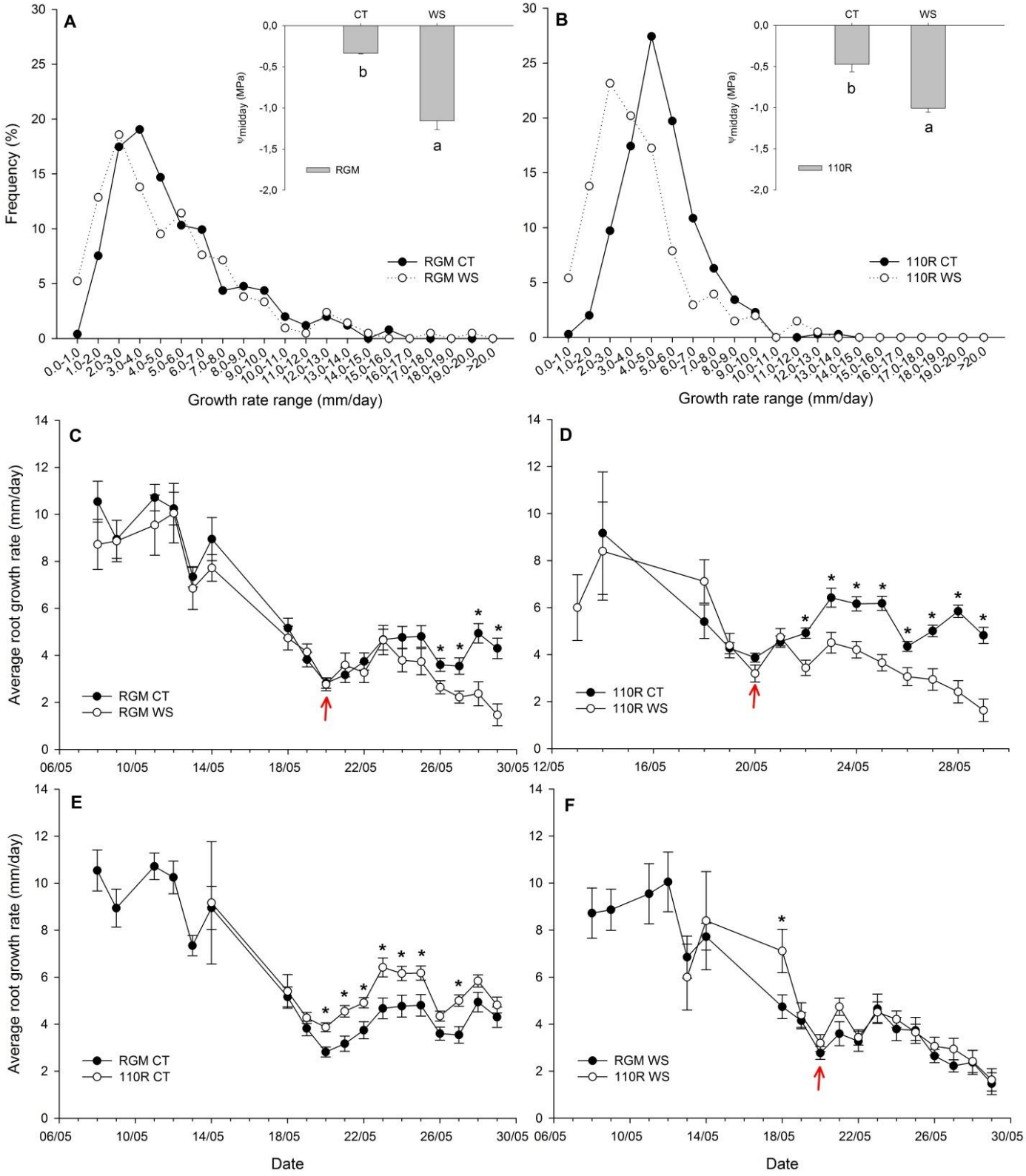

Figure 3.2 Root growth data and Ψ_{midday} of RGM and 110R under well-watered and water-stressed conditions, May 2015, stopped irrigation on 20th May 2015. A and B, individual root

growth rate distribution curves of RGM and 110R under well-watered and water-stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons (2015 and 2016 growing season). Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents $0.0 \leq$ growth rate < 1.0 mm/day). The frequency of root growth rates within each window for both genotypes (A, RGM; B, 110R) was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions involved is: n-RGM CT = 252, n- RGM WS = 210, n-110R CT = 351, n-110R WS = 203. Insets in A and B are Ψ_{midday}, values are mean ± SE, and different letters represent values that are significantly different ($n = 3$, t test, $p < 0.05$). C and D, comparison of average daily root growth rate from well-watered and water-stressed plants for both varieties (C, RGM; D, 110R). The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-RGM CT = 5-18, n-RGM WS = 7-15; n-110R CT = 3-36, n-110R WS = 4-20). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrows in C and D indicate the start of water stress treatment. E and F, comparison of average daily root growth rate of RGM and 110R for both CT and WS treatments. Values in E and F are the same as in C and D. To assess the effect of genotype on root growth, t test was also used to compare the root growth rate of RGM and 110R for each day under either well-watered (E) or water-stressed (F) conditions. As well, asterisks represent values that are significantly different at a confidence interval of 95%. Red arrow in F indicates the start of water stress treatment.

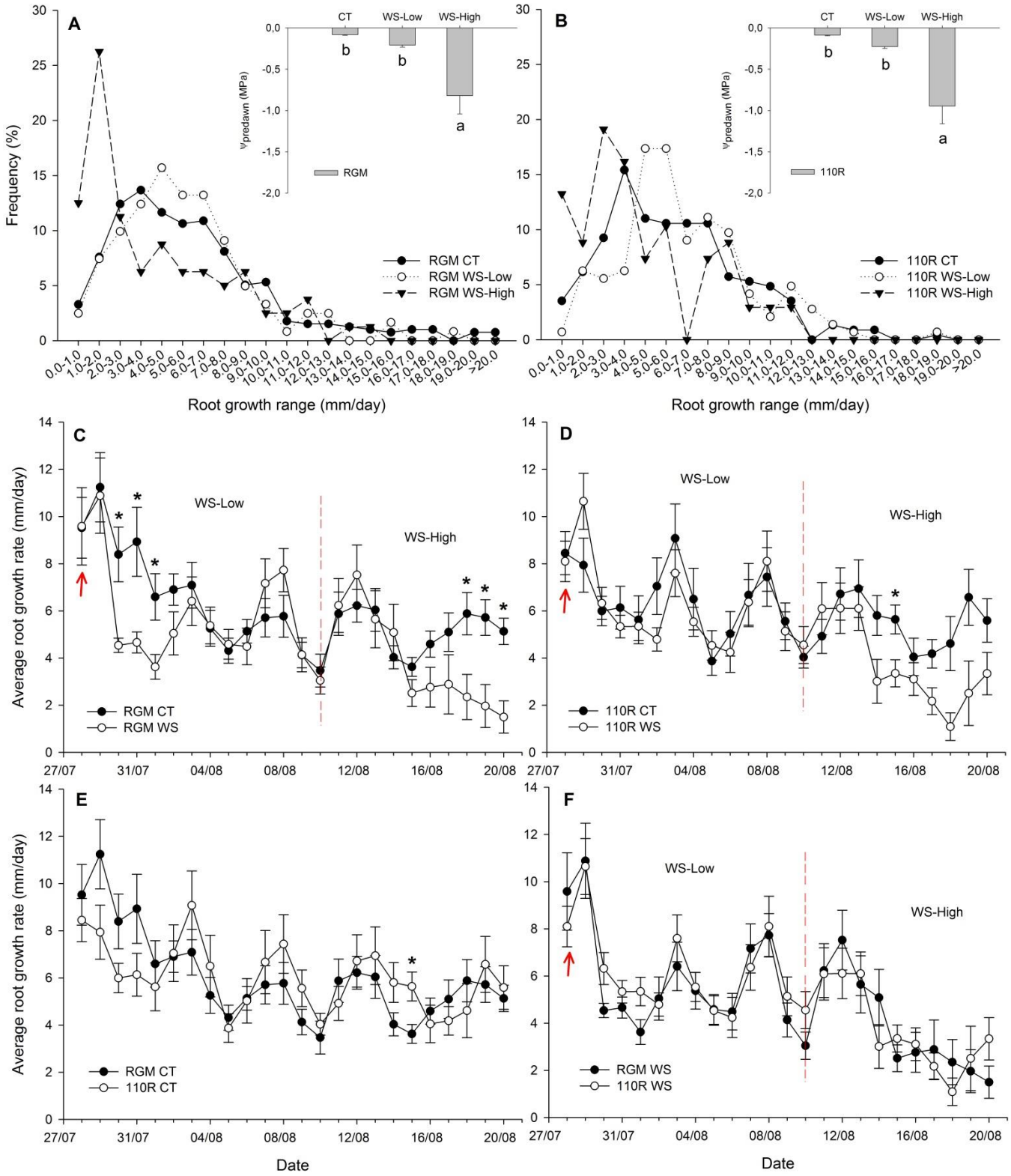

Figure 3.3 Root growth data and $\Psi_{predawn}$ of RGM and 110R under well-watered and water-stressed conditions, July-August 2015, stopped irrigation on 28th July 2015. A and B, individual

root growth rate distribution curves of RGM and 110R under well-watered and water-stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons. Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents 0.0 ≤ growth rate < 1.0 mm/day). The frequency of root growth rate within each window for both genotypes (A, RGM; B, 110R) was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions involved is: n-RGM CT = 395, n- RGM WS-Low = 212, n- RGM WS-High = 80; n-110R CT = 227, n-110R WS-Low = 144, n-110R WS-High = 68. Insets in A and B are $\Psi_{predawn}$, values are mean ± SE, and different letters represent values that are significantly different (for RGM, n-CT = 15, n-WS-Low = 6, n-WS-High = 6; for 110R, n-CT = 12, n-WS-Low = 6, n-WS-High = 6, $p < 0.05$). The number of biological replicates was 4 and 3 for CT and WS, respectively, for RGM, and the number of biological replicates was 3 for 110R. $\Psi_{predawn}$ was measured four times during the period of drought treatment to monitor the level of water stress treatment, and the plants were harvested when the average $\Psi_{predawn}$ dropped to around -1 MPa on the 20th of August. C and D, comparison of average daily root growth rate of well-watered and water-stressed plants for both varieties (C, RGM; D, 110R). The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-RGM CT = 11-26, n-RGM WS = 6-11; n-110R CT = 5-17, n-110R WS = 4-12). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrows in C and D indicate the start of water stress treatment. The dashed-line in C and D is the division of low and high level of water stress based on the $\Psi_{predawn}$ measured. E and F, comparison of average daily root growth rate of RGM and 110R for both CT and WS treatments. Values in E and F are the same as in C and D. To assess the effect of genotype on root growth, t test was also used to compare the root growth rate of RGM and 110R for each day under either well-watered (E) or water-stressed (F) conditions. As well, asterisks represent values that are significantly different at a confidence interval of 95%. Red arrow in F indicates the start of water stress treatment.

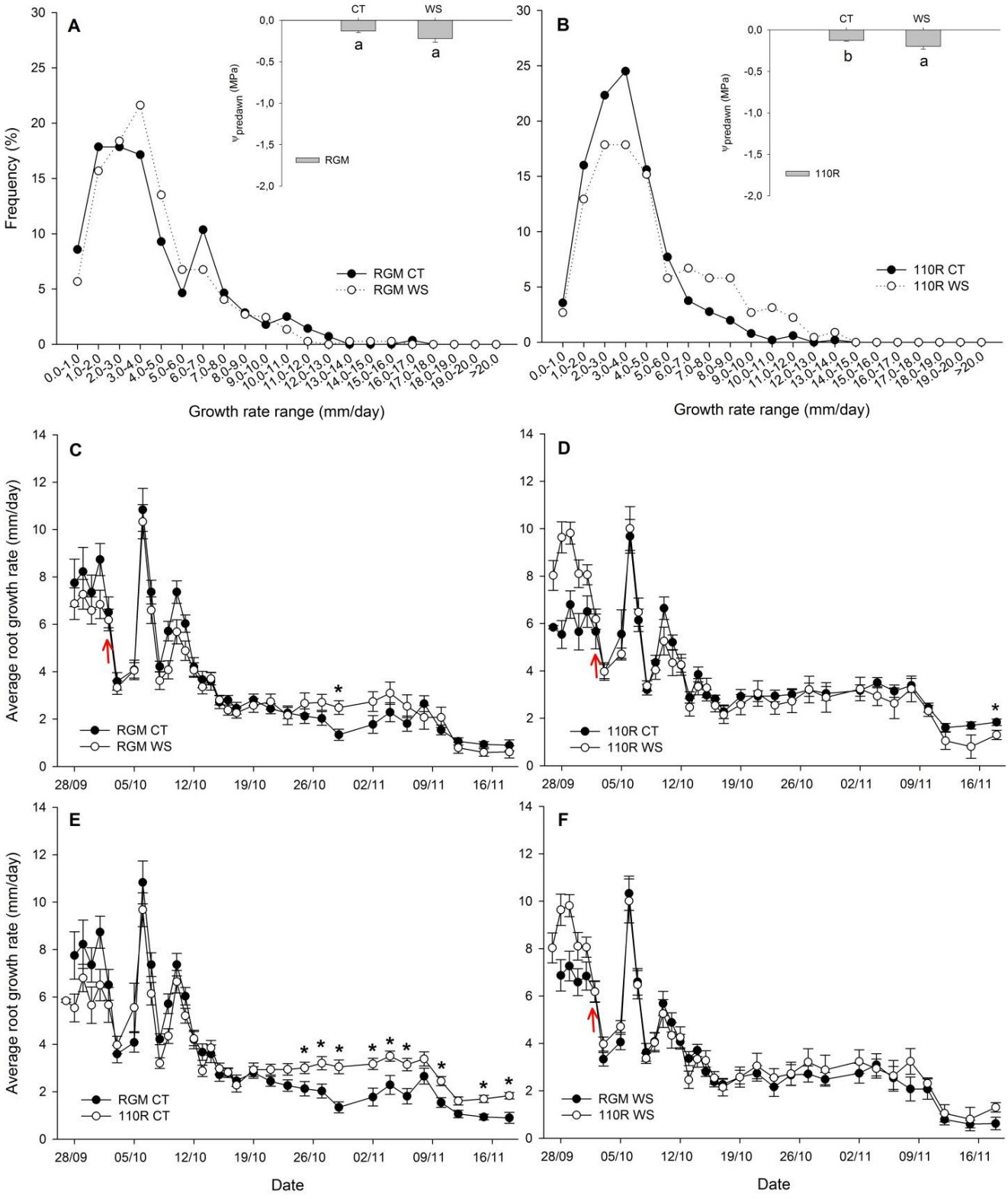

Figure 3.4 Root growth data and $\Psi_{predawn}$ of RGM and 110R under well-watered and water-stressed conditions, September-November 2015, stopped irrigation on 2nd October 2015. A and B, individual root growth rate distribution curves of RGM and 110R under well-watered and water-

stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons. Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents 0.0 ≤ growth rate < 1.0 mm/day). The frequency of root growth rate within each window for both genotypes (A, RGM; B, 110R) was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions involved is: n-RGM-CT = 280, n- RGM-WS = 370; n-110R-CT = 506, n-110R-WS = 224. Insets in A and B are $\Psi_{predawn,}$ values are mean ± SE, and different letters represent values that are significantly different (for RGM, n-CT = 12, n-WS = 10; for 110R, n-CT = 15, n-WS = 12, $p < 0.05$). The number of biological replicates was 3 for both CT and WS for RGM, and the number of biological replicates was 4 and 3 for CT and WS, respectively, for 110R. $\Psi_{predawn}$ was measured four times during the period of drought treatment to monitor the level of water stress treatment. C and D, comparison of average daily root growth rate of well-watered and water-stressed plants for both varieties (C, RGM; D, 110R). The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-RGM-CT = 5-10, n-RGM-WS = 4-14; n-110R-CT = 2-25, n-110R-WS = 2-10). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrows in C and D indicate the start of water stress treatment. E and F, comparison of average daily root growth rate of RGM and 110R for both CT and WS treatments. Values in E and F are the same as in C and D. To assess the effect of genotype on root growth, t test was also used to compare the root growth rate of RGM and 110R for each day under either well-watered (E) or water-stressed (F) conditions. Asterisks in E represent values that are significantly different at a confidence interval of 95%. Red arrow in F indicates the start of water stress treatment.

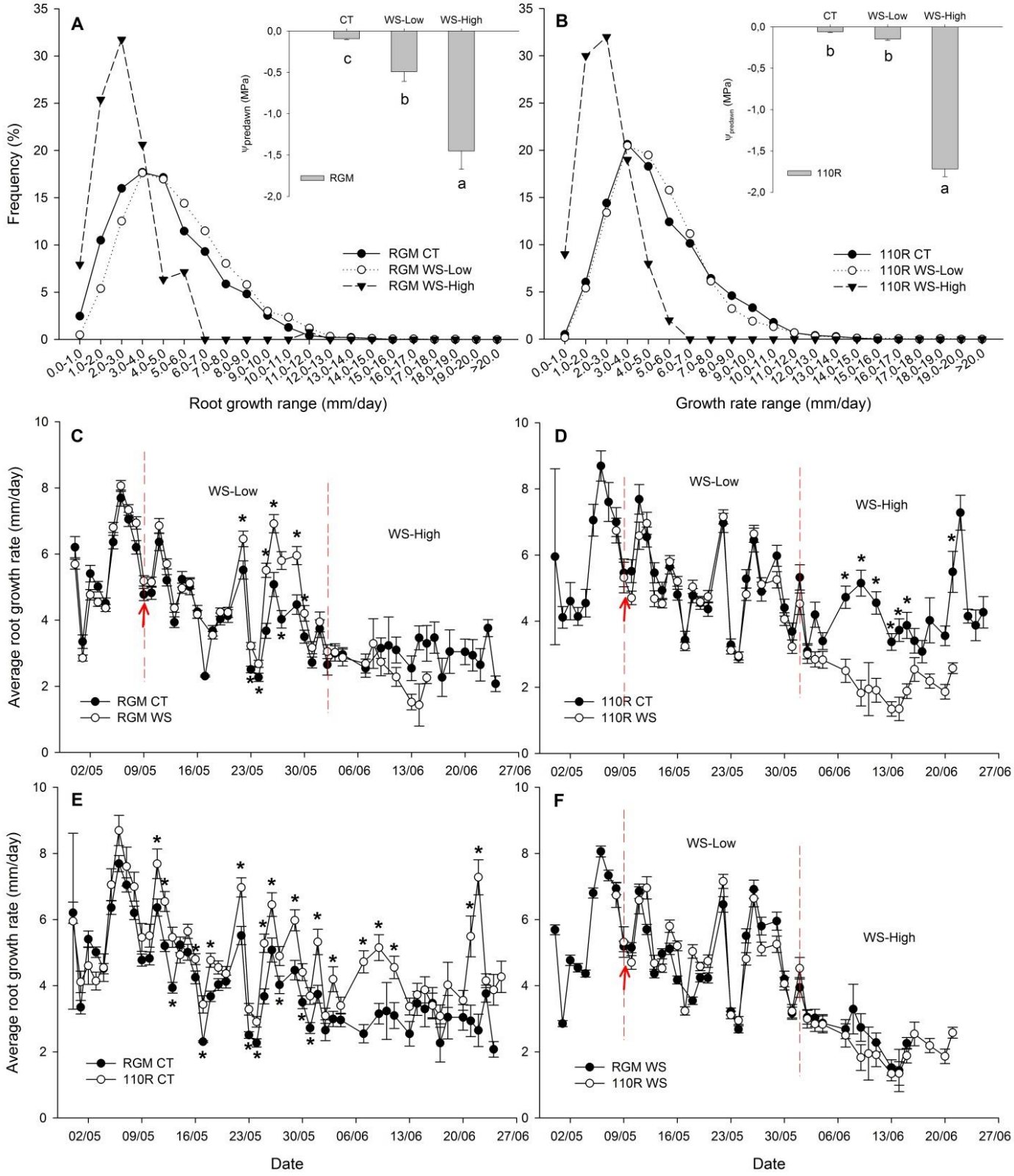

Figure 3.5 Root growth data and $\Psi_{predawn}$ of RGM and 110R under well-watered and water-stressed conditions, May-June 2016, stopped irrigation on 9th May 2016. A and B, individual root

growth rate distribution curves of RGM and 110R under well-watered and water-stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons. Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents $0.0 \leq$ growth rate < 1.0 mm/day). The frequency of root growth rate within each window for both genotypes (A, RGM; B, 110R) was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions involved is: n-RGM-CT = 2764, n- RGM-WS-Low = 3914, n- RGM-WS-High = 126; n-110R-CT = 1804, n-110R-WS-Low = 1889, n-110R-WS-High = 100. Insets in A and B are $\Psi_{predawn,}$ values are mean ± SE, and different letters represent values that are significantly different (for RGM, n-CT = 11, n-WS-Low = 9, n-WS-High = 5; for 110R, n-CT = 9, n-WS-Low = 2, n-WS-High = 9, $p < 0.05$). Water stress was achieved by stopping the irrigation completely. Therefore, with time going on, the drying-down process will create different levels of drought stress. Plants were sacrificed randomly for sampling along the period of drought treatment in order to get root samples with various stress levels and $\Psi_{predawn}$ was determined before each sampling. So the number of replicates in each category (CT, WS-Low and WS-High) actually corresponds to the number of samples in each group. C and D, comparison of average daily root growth rate of well-watered and water-stressed plants for both varieties (C, RGM; D, 110R). The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-RGM-CT = 3-125, n-RGM-WS = 2-192; n-110R-CT = 2-83, n-110R-WS = 2-134). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrows in C and D indicate the start of water stress treatment. The dashed-line in C and D is the division of low and high level of water stress based on $\Psi_{predawn}$ measured. E and F, comparison of average daily root growth rate of RGM and 110R for both CT and WS treatments. Values in E and F are the same as in C and D. To assess the effect of genotype on root growth, t test was also used to compare the root growth rate of RGM and 110R for each day under either well-watered (E) or water-stressed (F) conditions. Asterisks in E represent values that are significantly different at a confidence interval of 95%. Red arrow in F indicates the start of water stress treatment.

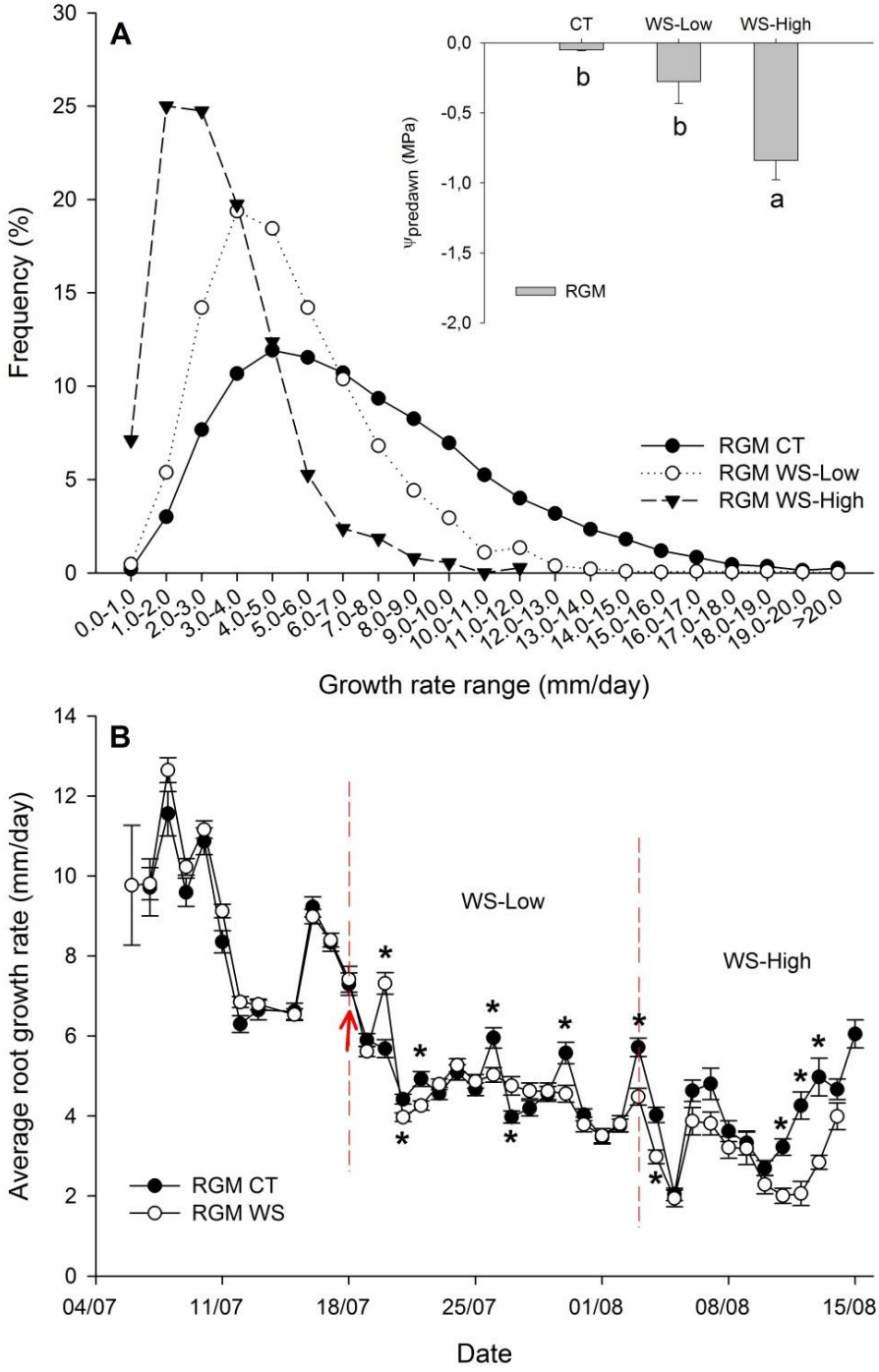

Figure 3.6 Root growth data and $\Psi_{predawn}$ of RGM for under well-watered and water-stressed conditions, July-August 2016, stopped irrigation on 18th July 2016. A, individual root growth rate distribution curves of RGM under well-watered and water-stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons. Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents 0.0 ≤ growth rate < 1.0 mm/day). The

frequency of root growth rate within each window was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions involved is: n-RGM-CT = 6954, n- RGM-WS-Low = 2380, n- RGM-WS-High = 380. Inset in A is $\Psi_{predawn}$, values are mean ± SE, and different letters represent values that are significantly different (n-RGM-CT = 15, n-RGM-WS-Low = 7, n- RGM-WS-High = 14, $p < 0.05$). Water stress treatment was achieved by stopping the irrigation completely. Therefore, with time going on, the drying-down process will create different levels of drought stress. Plants were sacrificed randomly for sampling along the period of drought treatment in order to get root samples with various stress levels and $\Psi_{predawn}$ was determined before each sampling. So the number of replicates in each category (CT, WS-Low and WS-High) actually corresponds to the number of samples in each group. B, comparison of average daily root growth rate of well-watered and water-stressed plants. The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-RGM-CT = 23-289, n-RGM-WS = 3-429). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrow in B indicates the start of water stress treatment. The dashed-line in B is the division of low and high level of water stress based on $\Psi_{predawn}$ measured.

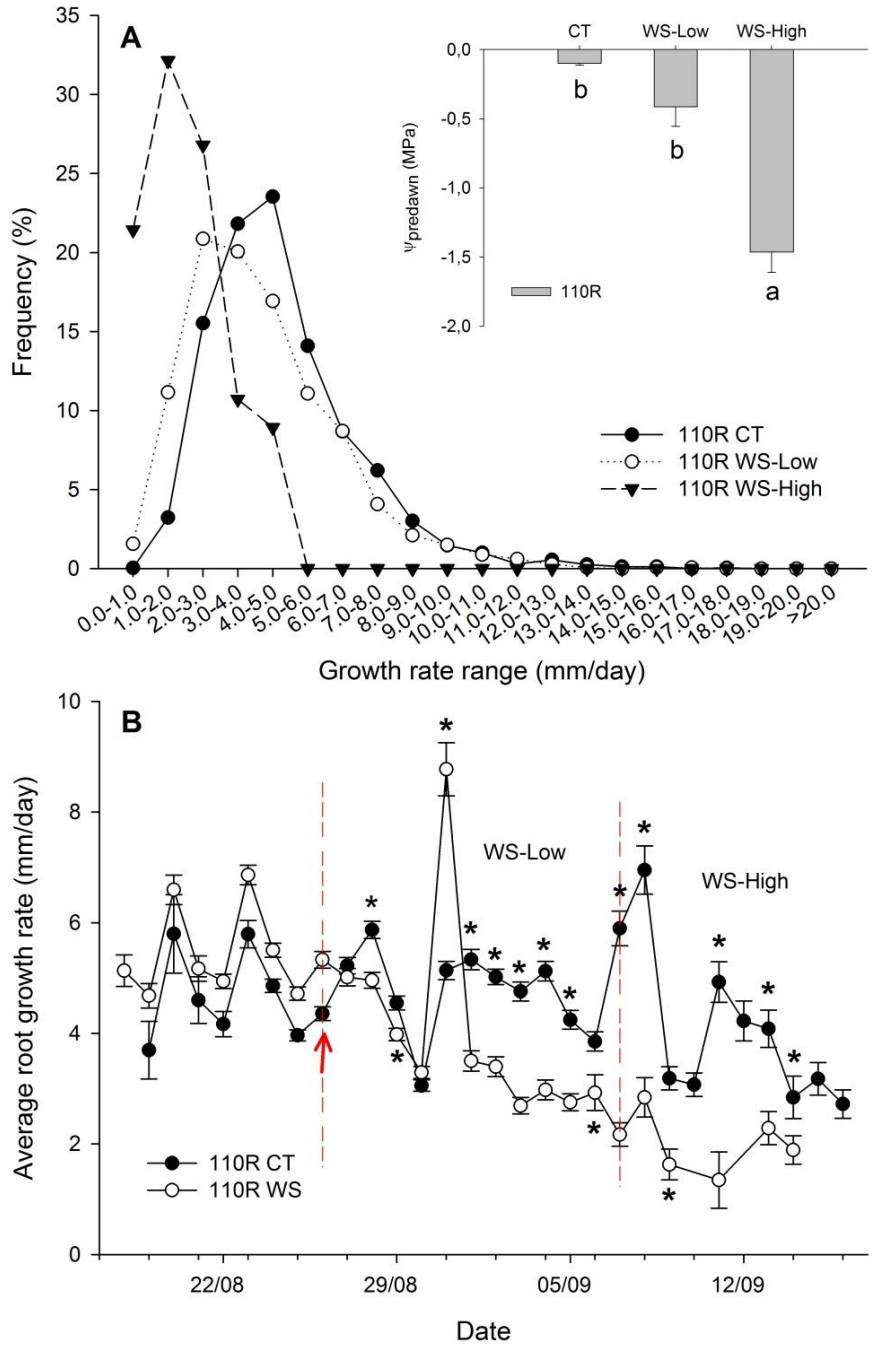

Figure 3.7 Root growth data and $\Psi_{predawn}$ of 110R under well-watered and water-stressed conditions, August-September 2016, stopped irrigation on 26th August 2016. A, individual root growth rate distribution curves of 110R under well-watered and water-stressed conditions. The scale of root growth rate range was determined by all daily root growth rates measured in all the experiments across these two growing seasons. Furthermore, the range of root growth rate was divided into 20 small windows of 1 mm/day (e.g., 0.0-1.0 represents 0.0 ≤ growth rate < 1.0 mm/day). The frequency of root growth rate within each window was calculated based on all daily growth rates observed during the period of the experiment. Number of root portions

involved is: n-110R-CT = 2384, n- RGM-WS-Low = 1249, n- RGM-WS-High = 56. Inset in A is $\Psi_{predawn}$, values are mean ± SE, and different letters represent values that are significantly different (n-110R-CT = 9, n-110R-WS-Low = 6, n- 110R-WS-High = 4, $p < 0.05$). Water stress treatment was achieved by stopping the irrigation completely. Therefore, with time going on, the drying-down process will create different levels of drought stress. Plants were sacrificed randomly for sampling along the period of drought treatment in order to get root samples with various stress levels and $\Psi_{predawn}$ was determined before each sampling. So the number of replicates in each category (CT, WS-Low and WS-High) actually corresponds to the number of samples in each group. B, comparison of average daily root growth rate of well-watered and water-stressed plants. The average root elongation rate of each day was calculated by making the mean of all root growth data collected on that day for both CT and WS. Values are mean ± SE (n-110R-CT = 6-187, n-RGM-WS = 4-224). To evaluate the effect of drought on root growth, t test was used to compare the root growth rate of CT and WS for each day. Asterisks represent values that are significantly different at a confidence interval of 95%. Red arrow in B indicates the start of water stress treatment. The dashed-line in B is the division of low and high level of water stress based on $\Psi_{predawn}$ measured.

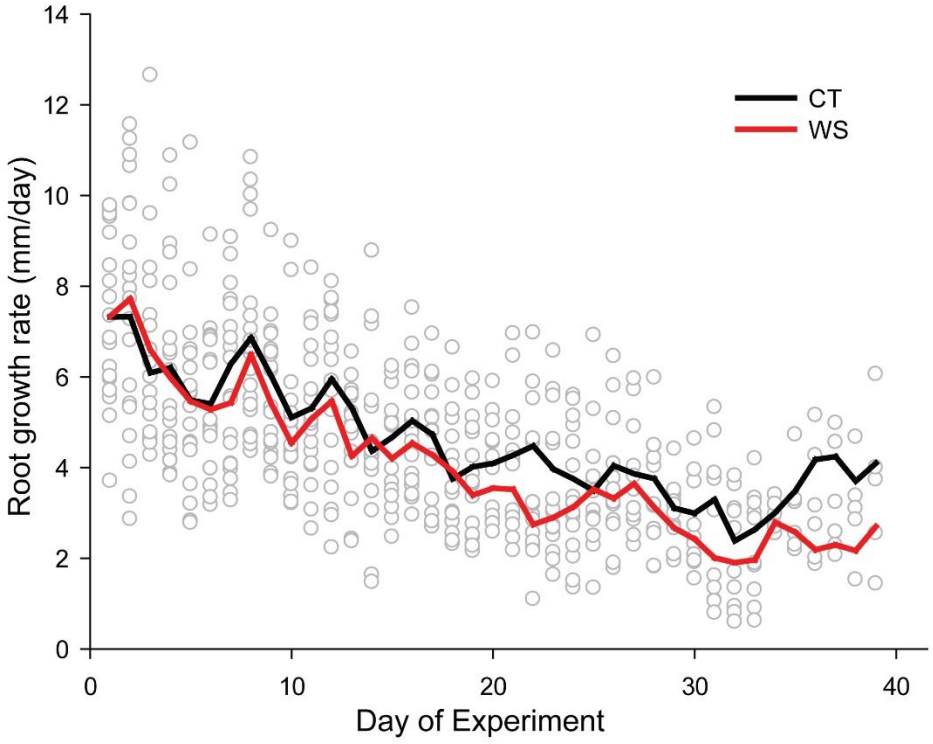

Figure 3.8 Changes of root growth rate in function of day of experiment. Grey circles represent average daily root growth rate for each condition (four conditions in total: 110R CT, 110R WS, RGM CT, RGM WS). Black and red lines represent the average growth rate of CT and WS, respectively, for each day of experiment. Data include all measurements from all the experiments conducted in both growing seasons.

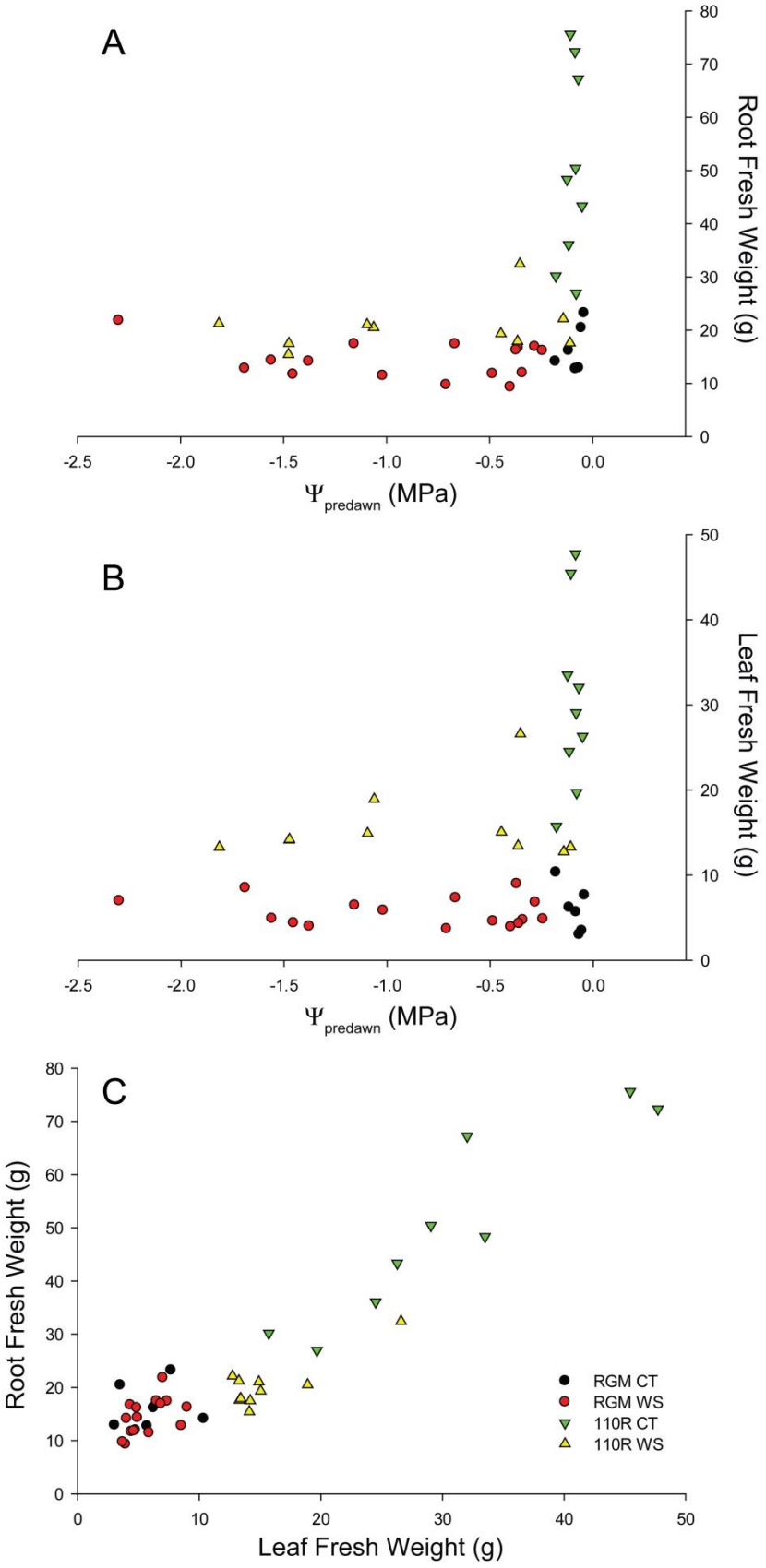

Figure 3.9 Relationship between root/leaf fresh weight and pre-dawn leaf water potential. A, relationship between root system fresh weight and $\Psi_{predawn}$. B, relationship between leaf fresh weight and $\Psi_{predawn}$. C, relationship between leaf fresh weight and root system fresh weight. Data were collected from the July-August 2016 experiment for RGM and from the August-September 2016 experiment for 110R. Scattered dot plots were made with all individual plants measured.

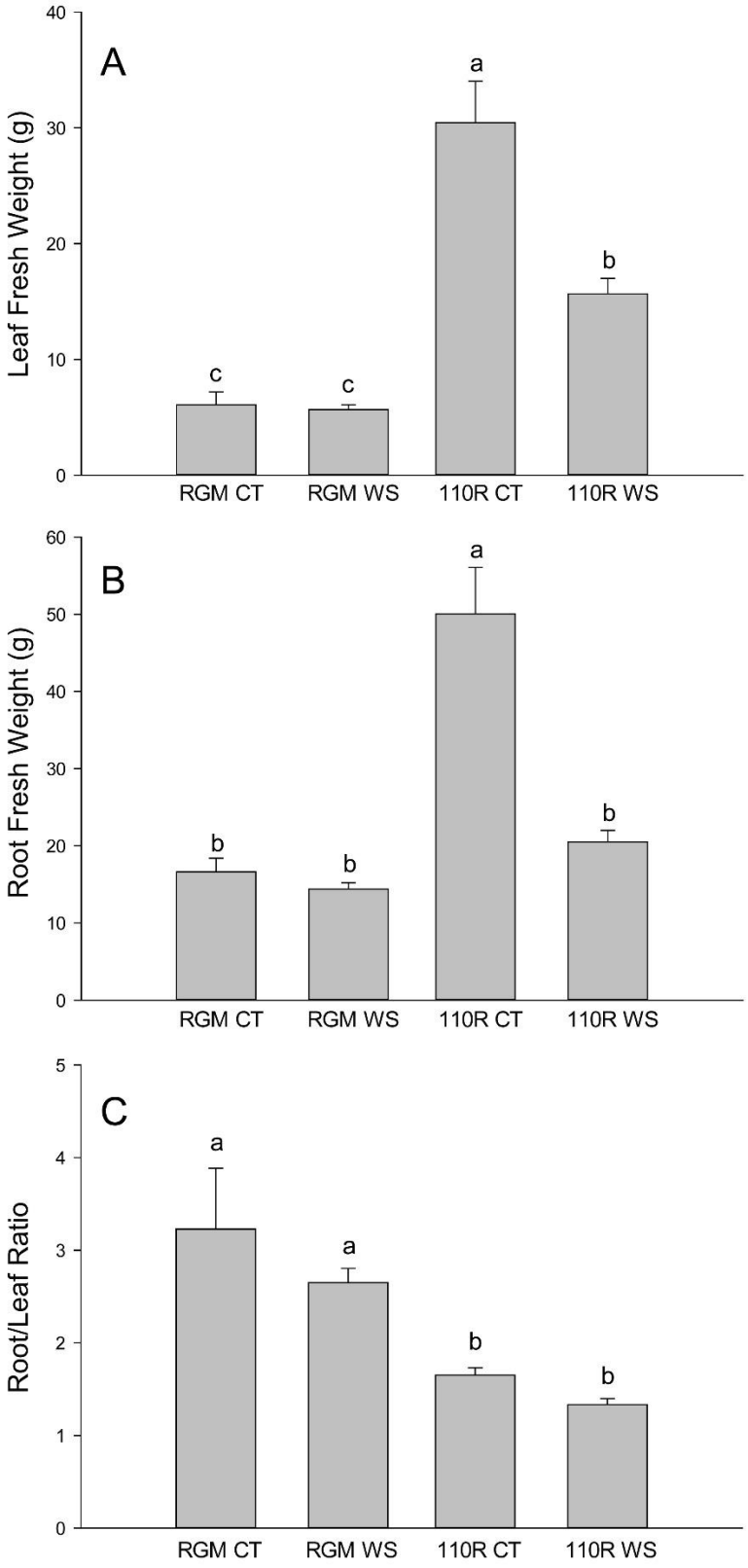

Figure 3.10 Changes in average leaf and root system fresh weight as well as root/leaf ratio in response to water stress. A, average leaf fresh weight under well-watered and water-stressed conditions for RGM and 110R. B, root system fresh weight under well-watered and water-stressed conditions for RGM and 110R. C, root/leaf ration under well-watered and water-stressed conditions for RGM and 110R. Data were collected from the July-August 2016 experiment for RGM and from the August-September 2016 experiment for 110R. Values are mean ± SE and different letters represent values that are significantly different (n-RGM CT = 6, n-RGM WS = 16, n-110R CT = 9, n-WS = 10, $p < 0.05$).

3.3.4 Changes in root and leaf growth mass in response to water stress

Root and leaf fresh weight was recorded for RGM in the July-August 2016 experiment and for 110R in the August-September experiment. For RGM, individual root and leaf fresh weight did not change with decreasing $\Psi_{predawn}$; for 110R, individual root and leaf fresh weight was reduced with decreasing $\Psi_{predawn}$, but the level of water stress treatment did not alter the fresh weight of root system and leaves (A and B in Figure 3.9). Under both well-watered and water-stressed conditions, 110R showed higher level of vegetative growth (A and B in Figure 3.9). Leaf fresh weight seemd to be positively correlated with root fresh weight, as plant with higher root mass also had higher leaf mass (C in Figure 3.9). On average, water stress did not influence root or leaf mass or root/leaf ratio for RGM, while it significantly decreased both root and leaf growth in 110R, but did not cause any difference in terms of root/leaf ratio (Figure 3.10). Under well-watered conditions, 110R showed much higher root and leaf mass growth (A and B in Figure 3.10). Water stress significantly decreased root mass growth in 110R. As a result, no difference was observed between RGM and 110R in root mass. Water stress decreased leaf mass growth in 110R as well, but leaf mass was still higher in 110R compared to RGM (A and B in Figure 3.10). Root/leaf ratio was significantly higher in RGM than in 110R under both well-watered and water-stressed conditions (C in Figure 3.10).

3.3.5 Changes in root anatomy in response to water stress

Changes in root anatomy, particularly in the exodermis and endodermis, in response to water stress have been observed in RGM and 110R. Increased degree of suberization in both exodermis and endodermis of root tips were observed associated with water deficit. In general, we observed that the development of root primary growth can be categorized into several stages, e.g. non-differentiated, differentiated (E-L), suberization of exodermis (B-D), partial suberization of endodermis (I-J), complete suberization of endodermis (K-L) (Figure 3.11). The occurrence of suberization and vascular tissue in fine roots from water-stressed plants was closer to the tip. For the moment, with the microscopy technique we adopted we did not find differences in the suberization pattern or in the structure between the two genotypes. Therefore, only the anatomical structure of 110R is displayed.

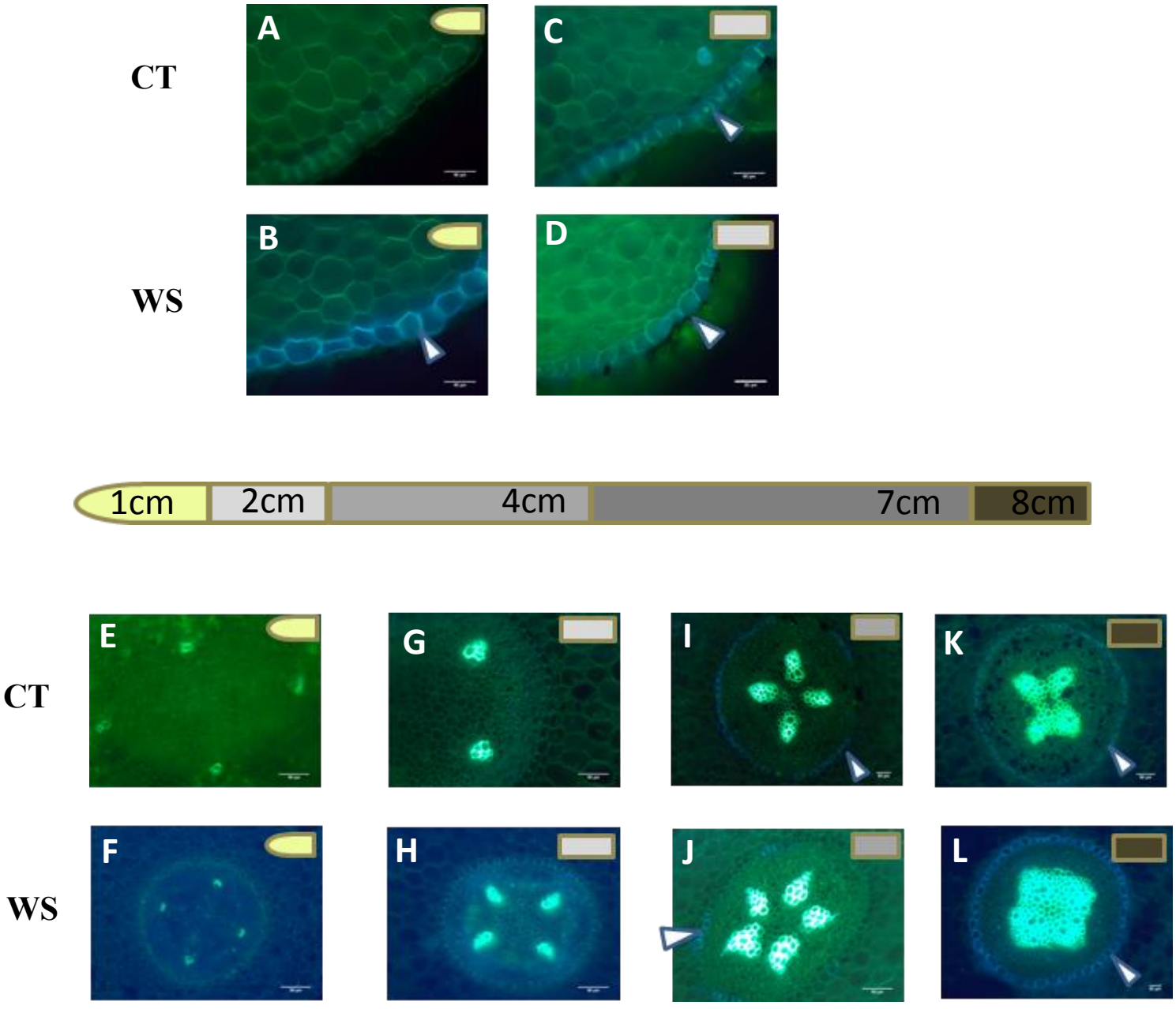

Figure 3.11 Patterns of suberization in exodermis and endodermis of root sections at different distances from root tips for 110R from the July-August 2015 experiment. White arrows indicate suberization. Bars = 50 µm

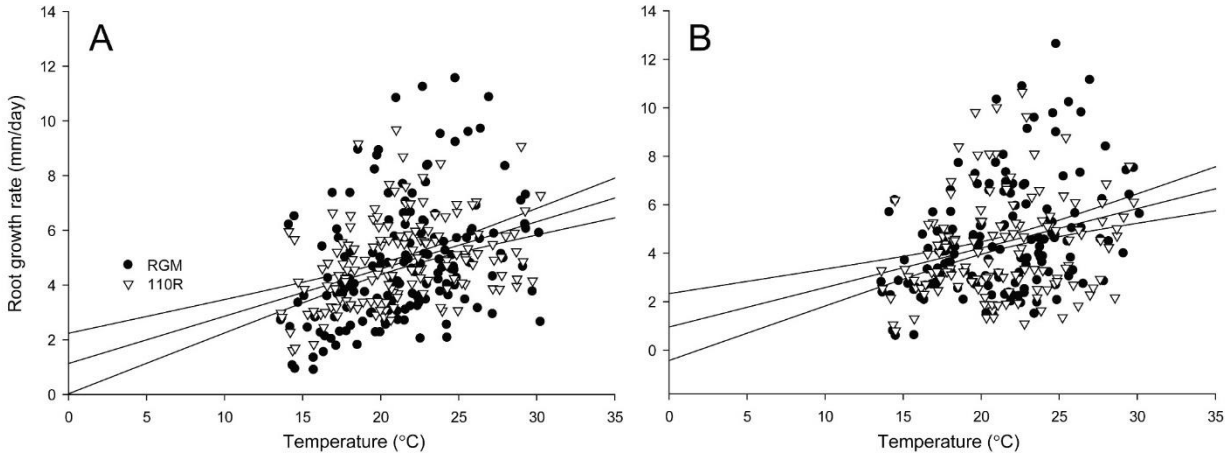

Figure 3.12 Relationship between root growth rate and soil temperature under well-watered (A) and water-stressed (B) conditions for RGM and 110R. Average daily root growth rate for each condition and average daily soil temperature are used for the plots. Data include all the measurements from all the experiments across two growing seasons. Regression lines in both figures represent the 95% confidence intervals.

3.3.6 Influence of soil temperature on root growth rate

For both RGM and 110R, average root growth rates from all the experiments performed were plotted against average daily soil temperature to explore the impact of temperature on root growth (Figure 3.12). Panel A displays the relationship between average daily root growth rate and average daily soil temperature for RGM and 110R under well-watered conditions, while panel B displays the data under water-stressed conditions. Generalized linear regression (GLM) was used to analyse the potential correlation between root growth rate and soil temperature. Even though the scattered points are very noisy, significant correlation at $p < 0.001$ was observed for both well-watered and water-stressed conditions, which suggests that average root growth rate is higher when soil temperature is high under both well-watered and water-stressed conditions.

3.4 Discussions

3.4.1 Measuring root growth in cylinder rhizotrons

In general, the cylinder rhizotron used in our experiment proved to be a simple and efficient method to study root growth. In addition to its non-destructive property, the relative large volume and deep depth provided a growing environment much closer to field conditions than growing in hydroponic solutions or hydroponic based rhizotrons, or even small pots. During the drying-down process when plants were subjected to water stress treatment, the sand in the top layer dried out rapidly, while the sand in the middle and especially at the bottom of the rhizotron can retain moisture for longer time. However, unlike in the field, there is no available water source from the

deeper soil layer for plants growing in rhizotrons. Therefore, the circumstance where roots grow deeper in the soil to search for available water does not apply for the container rhizotron setup. Moreover, root growth will be restricted as soon as it reaches the bottom of the rhizotron. Recording the position of roots with marker pen every day and in the end measuring root length and calculating root growth is an easy way to approach to assess growth, but compared with computerized image acquisition and analysis, this is a very laborious and tedious method. Although the data collected (root length and root growth rate) are precise and accurate, it is not possible to assess parameters like root diameter, root density, root surface area, or root branching and distribution patterns. Another disadvantage in this rhizotron method is that only a small part of the whole root system can be viewed through the transparent wall. Many researches have evaluated various methodologies and technologies available in root studies and inevitably they are all attributed with both advantages and disadvantages (e.g., Taylor *et al.* 1990, Fiorani and Schurr 2013, Judd *et al.* 2015, Mohamed *et al.* 2017).

Nevertheless, the purposes of this project did not only lie in studying root growth as what we are interested in is also to discover the relationship between root growth and root water uptake as well as between root growth and aquaporin gene expression. Taken together, the rhizotron approach we adopted is sufficient enough to obtain the information we need in terms of root growth rate while allowing for sampling of individual roots with known growth rate.

3.4.2 Root growth

In our study, we constantly observed that root growth rate fluctuated enormously from one day to another during the period of each experiment with roots growing at 3-5 mm/day accounting for the largest proportion and with average root elongation rate in the range of 1-12 mm/day under well-watered conditions. To date, root growth rate has not been extensively investigated in *Vitis* and we were not able find similar results in the literature. Nonetheless, some early studies are available in other species under both field and greenhouse conditions.

The dynamics of root growth are the result of the interaction between the internal growth mechanisms and the external impacts of environmental conditions (Walter and Schurr 2005, Walter *et al.* 2009). Plant growth is highly responsive to their surrounding environment. Carbohydrate availability and partitioning are key factors determining root growth as this growth is an energy-dependent process and requires either photosynthate or starch reserves (Ritchie and Dunlap 1980, Clarke *et al.* 2015). Fluctuation of root growth rate during the period of the experiment can be attributed to several elements. First of all, temperature is a major player in affecting the growth and development of root tissues. Teskey and Hinckley (1981) looked into root growth of white oak (*Quercus alba* L.) in an oak-hickory forest using an observation chamber (field rhizotron) during a period of 18 months across two growing seasons. Root elongation rate varied seasonally in function of soil temperature and moisture with a maximum growth rate of 5.2 mm/day observed. Greenhouse studies revealed average taproot elongation rate between 34.5 and 46.5 mm/day of soybeans planted in vermiculite, while in field experiment,

root elongation rate decreased to 23 mm/day (Kaspar 1982) or even lower to 17 mm/day for soybean and 13 mm/day for maize (*Zea mays* L.) (Allmaras *et al.* 1975). As shown in our experiment, root growth was maintained at a lower speed in the October-November growing period in 2015 growing season. Kaspar (1982) also observed that soybeans had lower taproot elongation rates in early winter than in early summer. And he speculated that decreased solar radiation, shorter natural day length, and slightly cooler glasshouse temperatures could be the reasons that caused reduced root growth in early winter. In another greenhouse experiment conducted in durum wheat (*Triticum turgidum* L. var. durum) (Simane *et al.* 1993), relative growth rate declined throughout the growing season (36 to 136 days after emergence) irrespective of the treatments (control or different timing of moisture stress).

In grapevine, it has been shown that optimum root growth occurs at around 30°C (Richards 1983). However, this value may change depending on the genotype (Clarke *et al.* 2015). In general, grapevine roots exhibit a very distinct growth pattern observed in all different rootstocks (Delrot *et al.* 2001). Two predominant growing phases at flowering and harvest were found respectively, which possibly resulted from the temperature requirement and the sink demand related to the growth of shoots and roots (Van Zyl 1988, Delrot *et al.* 2001). Bud growth is promoted in early spring when soil temperature is lower than air temperature. Root growth is apparently delayed until the emerging of leaves who serve as active sinks for assimilates. Later on, berry growth will be competing with root growth from fruitset until harvest (Delrot *et al.* 2001).

In terms of the effects of soil temperature on grapevine growth, Woodham and Alexander (1966) have reported that bud-break and shoot development, shoot and root growth, and percent fruit-set were considerably higher at high root temperature (30 °C) than at low root temperature (11 °C) in Thompson Seedless grapevines. Likewise, Zelleke (1977) and Zelleke and Kliewer (1979) observed that root and shoot growth were significantly greater at 25 °C soil temperature than at 12°C soil temperature. Therefore, during the period of all the experiments, as temperature fluctuates from day to day and diurnally within one day as well, it is apparent that root growth rate will show fluctuations.

The growing degree days, calculated form the sum of air temperature higher than a certain threshold, is a criterion used to evaluate the extent of development in plant (Johnson and Thornley 1985). In grapevine, the base temperature for the calculation of growing degree days is set at 10 °C (Amerine and Winkler 1944). Pregitzer *et al.* (2000) pointed out that it would be reasonable to assume that the commencement and extent of root growth might be related to the cumulative heat sum of the soil. As the metabolic and catabolic processes in plants are temperature-sensitive, according to Clarke *et al.* (2015), the reason why roots grow faster in warmer soil could be resulted from a greater rate of root reserve carbohydrate catabolism at higher temperature, which will provide more energy and C skeleton to stimulate root growth. Moreover, Skene and Kerridge (1967) and Zelleke (1977) have reported that cytokinin content in grapevine roots was upregulated at higher temperature (30 °C and 25 °C, respectively) and suggested that the stimulated bud-break, shoot and root growth might be the result of cytokinin

activity. However, when temperature got to 35 °C, Gur *et al.* (1972) observed a reduced level in root and leaf cytokinin contents in apple tree, and root and shoot growth was slowed down as well at this temperature. Zelleke and Kliewer (1980) have reported greater uptake of mineral nutrients and synthesis of organic substances in Cabernet sauvignon growing at root temperature of 25 °C than at 12 °C, which to some extent could explain the increased level of cytokinin and consequently the stimulated bud-break, shoot and root growth. Zelleke and Kliewer (1980) also observed that at higher temperature, grapevine can translocate more nitrogenous substances to the above ground parts from the roots. More recently, Rogiers *et al.* (2014) have confirmed that elevated root-zone temperature at 22.6 °C ~ 24.8 °C from budburst to fruitset in Shiraz (*Vitis vinifera* L.) stimulated the mobilization of carbohydrate reserve in roots as well as the translocation of nitrogen and potassium to berry and petiole, and accelerated shoot growth and reproductive development (e.g., flowering, fruitset, véraison). In a similar experiment conducted by Clarke *et al.* (2015), stimulated root growth, root branching, mobilization of carbohydrate reserve in roots and canopy development have also been reported. Moreover, warmer soils have also enhanced the uptake of primary nutrients (e.g., N, P, K, Ca and B) by increased number and length of active roots. Experiment with ^{15}N isotope labelling provided evidence that higher N content in grapevines exposed to warmer soil temperature was resulted from improved nutrient absorption. However, notably, the concentration of other macronutrients in petioles was either lower (Mg and Na) or unchanged (Fe and Zn) from vines growing in warmer soils, which probably indicated a complex process of nutrient uptake or suggested that the optimal temperature for each element absorbed by roots is not the same.

In general, average root growth rate seems to be positively correlated with soil temperature under both well-watered and water-stressed conditions across all the experiments conducted in our study as shown in Figure 3.2. A summary of the ranges of soil temperature and average root growth rate of well-watered plants from all experiments performed during the two growing seasons is presented in Table 3.2, and in addition average soil temperatures for each month during the period of experiments across two growing seasons are presented in Figure 3.13. Apparently, we can see that root growth of both cultivars was markedly slowed down in the late growing season (mid-October to mid-November) of the year and soil temperature was obviously decreased. We assume that decreased temperature in autumn may be a key factor for decreased growth rate. Meanwhile, as a matter of fact, during the late growing season, coupled with reduced temperature is reduced irradiance from sunlight (e.g., reduced light intensity, shorter daytime, more cloudy days). Lower irradiance received by shoots may have slowed down root growth as well. Temperature changes in the experiments conducted in July-August 2015, May-June 2016, July-August 2016, and August-September 2016 fell in a similar range, and so did the fluctuations of the average root growth rate for both genotypes. Soil temperature seems relatively lower in May 2015, but root growth rate is consistent with the other experiments. An explanation for this could be that roots growing faster in spring and early summer is a behavior based on their phenology and 17.1-22.6 °C is a desirable range of temperature to activate dormant woody parts and to promote their growth and development. One thing noteworthy is that grapevine cuttings

were newly planted for each experiment within one growing season. Therefore, the comparison between experiments within one growing season does not equal to the pattern of seasonal variation in root growth observed under field conditions.

As reviewed earlier in chapter 2, photoreceptors are expressed in both dark and light grown roots; therefore, it is reasonable to assume that light can influence root growth through several ways. First of all, root growth can be affected by light when they are exposed directly in light. For example, in our experiment, when we measured root growth rate by marking the position of all visible roots on the transparent paper sticked to rhizotron every day, all roots were exposed to natural light for a very short period time of approximately one minute. Secondly, when roots are growing in darkness, possibly light can be transmitted through stem via vascular tissues. Thirdly, the above-ground parts sense light and communicate with roots via relevant signals and cause corresponding reactions. However, the consequences resulted from roots exposure to light can be neglected in our experiments because the duration of the exposure is very short. Nevertheless, even though the fluctuation of root growth rate may be to some extent caused by exposure to light, this influence is identical to all roots measured.

Light intensity received by shoots can be one important factor for the observed constant fluctuations in both individual and average root elongation rate. In viticulture, it is a common practice to ameliorate sunlight penetration and distribution through the canopy by choosing the appropriate trellis or training system and by optimized canopy management. Extensive studies have been carried out concerning the effect of light environment within canopy on canopy parameters and berry composition and quality (e.g., Kaps and Cahoon 1992, Dokoozlian and Kliewer 1995a, 1995b, Keller *et al.* 1998a, 1998b). But not a lot of researches are available on the responses of root growth and development to changes in light intensity received by the canopy due to sampling difficulties of underground parts in viticulture practice. Grechi *et al.* (2007) cultured cuttings of grapevine (*Vitis vinifera* L. cv Merlot) aeroponically and explored the impact of light regime and external nitrogen supply on plant C:N balance and biomass allocation between roots and shoots. High pressure sodium vapor lamps and shading cloth were used to modify the amount of natural light reaching the plants, and high irradiance and low irradiance conditions were achieved, respectively. Root biomass was significantly influenced by light availability. Compared with controlled plants (irradiance received on average: 8.4 mol PAR/m^2/day, PAR: Photosynthetic active radiation), root biomass of grapevines grown under high irradiance (on average: 13.8 mol PAR/m^2/day) was increased by 94%, while this parameter was reduced by 58% in plants grown under low irradiance (on average: 5.3 mol PAR/m^2/day). The amount of nitrogen accumulated per organ was investigated in leaves, stems, trunk, roots, as well as the whole plant under different light regimes. High irradiance significantly increased the amount of nitrogen accumulated per organ in roots.

Root is devoted to mineral nutrient acquisition and is the first organ to sense and signal mineral starvation (Hermans *et al.* 2006). Nutrient availability can impose a profound impact on the growth of primary root, lateral root formation and elongation, angle and diameter of roots, and

root hairs, and ultimately will affect the architecture of the whole root system which is a determinant of how plant can efficiently adapt to environmental constraints (Gruber *et al*. 2013). For instance, reduced nitrogen availability to Arabidopsis seedlings fastens the elongation of primary roots while lateral roots formation remains constant under various concentrations of nitrate supply. On the contrary, under limited phosphorous availability, primary root growth is slowed down while lateral root density is significantly increased. Both high nitrogen and high phosphorous supply suppress lateral root elongation (Williamson *et al.* 2001, López-Bucio *et al.* 2002). Limited sulphur supply favors a more branched root system in Arabidopsis (Kutz *et al.* 2002). Even though potassium and magnesium deficiencies promote the accumulation of sugars and starch in young leaves, they are not available for root growth (Cakmak *et al.* 1994). In grapevine, deprivation of nitrogen stimulated root growth by increasing root biomass by 51% of that of the control plants. However, this increase was at the expense of the above-ground growth (Grechi *et al.* 2007). Conversely, excessive supply of certain ions can expose plant to salt stress or ion toxicity which reduces plant growth in general. For example, tap root growth of cotton (*Gossypium hirsutum* L.) seedlings is inhibited at high NaCl concentrations (Cramer *et al.* 1986).

In our experiments, all well-watered plants were irrigated with a balanced nutrient solution. Therefore, neither the fluctuation of root growth during each growing season of the experiment, nor the considerably reduced root growth speed in the later stage of each growing season, nor the remarkably declined root growth are accounted for nutrient deficiency or surplus.

Table 3.2 Summary of minimum and maximum average root growth rate and soil temperature

Period	Range of average root growth rate (mm/day)		Range of soil temperature (°C)
	RGM	110R	
8 May 2015-29 May 2015	2.8-10.7	3.8-9.2	17.1-22.6
28 July 2015-20 August 2015	3.5-11.2	3.9-9.1	19.9-29.8
19 October 2015-18 November 2015	0.9-2.8	1.6-3.5	14.4-18.9
30 April 2016-25 June 2016	2.1-7.7	2.9-8.7	14.1-30.2
7 July 2016-15 August 2016	2.0-11.6	N/A	21.2-30.2
18 August 2016-16 September 2016	N/A	2.7-7.0	20.2-29.1

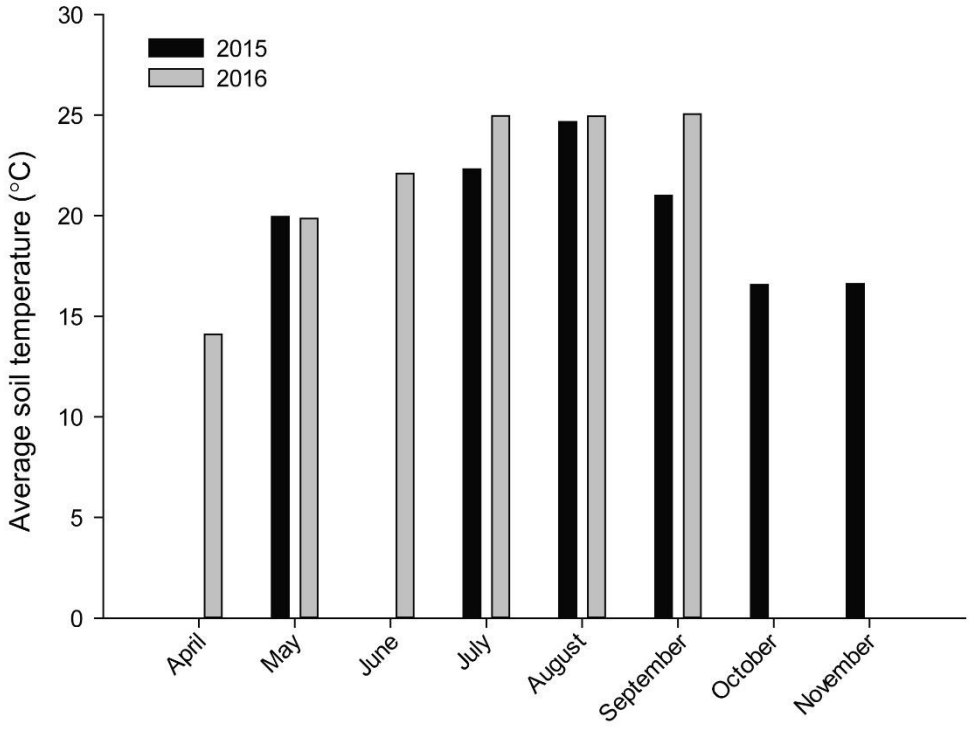

Figure 3.13 Average soil temperature for each month during the period of experiments for two growing seasons.

3.4.3 Impact of water stress

Root growth rate was down-regulated under water stress for both varieties, as can be seen from these two phenomena observed: individual root growth distribution curves shifted to the lower speed and average root elongation rate was decreased or significantly decreased. Root growth in terms of biomass of 110R was significantly inhibited by water stress treatment, while no change was observed in the root biomass of RGM under water stress (expressed in root fresh weight, panel A in Figure 3.9, and panel B in Figure 3.10). For both 110R and RGM, changes in leaf biomass under water stress exhibited the same pattern as changes in root biomass (expressed in leaf fresh weight, panel B in Figure 3.9, and panel A in Figure 3.10). In the end, for both RGM and 110R, no changes in root/leaf ratio were observed under water stress (panel C in Figure 3.10).

Although the exact mechanisms behind the responses of root system to water stress are not completely clear, significant progress has been made in understanding root growth and development under drought (Davies and Zhang 1991, Sharp and LeNoble 2002, Sharp *et al.* 2004, Xiong *et al.* 2006).

In general, when plants face water constraints, the development of root system is less inhibited than shoot growth, and may even be stimulated (e.g., in our experiment, second half of WS-Low in panel C Figure 3.5), ending up with increased root biomass and deeper rooting depth (Sharp

and Davis 1989). It has been reported that shoot growth can be limited even prior to the development of reduced water potentials in the aerial parts of the plant (Saab and Sharp 1989, Gowing *et al.* 1990). Maintenance of root growth at water potentials that are low enough to inhibit shoot growth is obviously advantageous to sustain an adequate water supply to the whole plant (Sharp *et al.* 2004). Meanwhile, reduced above-ground growth will decrease transpiration as well as demand for water supply (Hoogenboom *et al.* 1987), which provides another protective mechanism.

Rodrigues *et al.* (1995) have reported a stimulation of root growth in an herbaceous plant lupin (*Lupinus albus* L.) after 15 days of water stress treatment during flowering. Pre-dawn leaf water potentials of well-watered seedlings were maintained at around -0.1 MPa, while those were dropped to below -0.6 MPa in water-stressed seedlings. At the end of the treatment, water deficit induced a significant increase in the fine root length per unit soil volume and a slight increase in the fine root dry weight. The increase in fine root dry weight was more pronounced in deeper soil layers.

The continuous elongation of primary root has been observed in e.g. maize, soybean, cotton, and squash at water potentials which already limit shoot growth (Sharp *et al.* 1988, Spollen *et al.* 1993, Sharp *et al.* 2004). Primary roots still elongate at a considerable speed even when water potentials of the growth medium are lower than -1.5 MPa (Sharp *et al.* 2004). Notably, root elongation under water stress is maintained preferentially towards the apex (Sharp *et al.* 2004). Sharp *et al.* (1988) reported that at a water potential of -1.6 MPa of the growth medium, primary root elongation rate of maize (*Zea mays* L. cv WF9 x Mo17) was not modified in the first 3 mm of the root apex, but was progressively inhibited at more basal locations, and completely sopped at 7 mm from the apex. The length of the growing zone along the root was shortened with decreased water potential. Liang *et al.* (1997) reported similar results. Water-stressed plants tend to develop thinner roots (Sharp *et al.* 1988, Liang *et al.* 1997), which is consistent with our observations (data not collected). Similar responses in shoot and root growth to water deficit are observed in grapevine as well. Stevens *et al.* (1995) have reported a negative linear correlation between grapevine vegetative growth (expressed in pruning weight) and water stress index. Dry *et al.* (2000a, 2000b) have observed less decreased root growth than shoot growth under water deficit in grapevine.

Sustained root growth under drought is likely an adaptive response resulted from osmotic adjustment or an enhanced cell wall loosening capacity (Saab *et al.* 1992, Hsiao and Xu 2000, Chaves *et al.* 2002, Sharp *et al.* 2004). Besides, the plant hormone abscisic acid (ABA) has been proposed to be an important factor in the regulation of root growth and play an essential role in plant differential growth responses to water deficit (Saugy *et al.* 1989, Saab *et al.* 1990).

Osmotic adjustment and turgor maintenance in roots in response to water deficit could impact root : shoot partitioning patterns, and root and shoot growth via indirect action of root-produced plant growth regulators (Turner 1986, Ranney *et al.* 1991). Ranney *et al.* (1991) have reported

increased levels of soluble carbohydrates in water-stressed roots in cherry (*Prunus*) trees resulted primarily from an increase in sorbitol, which may contribute to a greater capacity of turgor maintenance. Voetberg and Sharp (1991) have reported a drastic progressive increase in proline concentration with decreasing water potentials in the first few millimeters of apex where the elongation was fully maintained in water-stressed roots in maize (*Zea mays* L. cv WF9 x Mol 7) seedlings. Compared with roots growing under higher water potential (-0.03 MPa), roots under a relative mild stress treatment (-0.2 MPa) had a 10-fold increase in proline concentration in the apex, while this increase reached to approximately 20-fold in roots under a more severe stress treatment (-1.6 MPa). Up-regulation in proline concentration accounted for up to 50% of the osmotic adjustment in root apex, while the other measured solutes, hexose, sucrose, various amino acids and potassium contributed only a small portion to the osmotic adjustment in root apex (Voetberg and Sharp 1991). Barcia *et al.* (2014) investigated the consequences of water deficit in wheat (*Triticum aestivum* L. cv 75 Aniversario) root growth under a range of water potentials (from -0.03 MPa to -1.2 MPa). Apparently, root elongation rate was reduced under water stress in correlation with the stress level. Under moderate water stress (-0.06 MPa), root osmolarity significantly increased with a great increase in the concentration of proline and total soluble carbohydrates. However, the role of proline in plant's response to water stress is under debate. According to Vendruscolo *et al.* (2007), accumulated high levels of proline in drought tolerant transgenic wheat were not a consequence of osmotic adjustment but rather a protective mechanism against oxidative stress. Shabala and Shabala (2011) pointed out that the predominant role of proline in plants subjected to drought is to protect cellular functions and organs, even though it may contribute slightly to osmotic adjustment. In grapevine, it has been reported that under water stress, although leaf growth was completely inhibited, turgor pressure was maintained at or above that of the controls by osmotic adjustment in leaves (Schultz and Matthews 1993). Furthermore, the authors demonstrated that the inhibition of leaf expansion under water stress was due to decreased cell-wall extensibility.

In addition to osmotic adjustment, changes in cell wall extensibility and cell wall proteins is another adaptive mechanism in response to water stress, as osmotic adjustment may not be sufficient to maintain turgor (Sharp *et al.* 2004). Wu *et al.* (1996) showed that at a low water potential of -1.6 MPa, acid-induced cell wall extensibility was greatly increased in the apical 5 mm of maize primary root, which is in agreement with sustained elongation in root apex reported by Sharp *et al.* (1988). Meanwhile, associated with increased cell wall extensibility was enhanced activity of cell wall extension protein-expansins and higher cell wall susceptibility to expansins. In contrast, acid-induced cell wall extension was largely decreased in the 5-10 mm region of root apex under water stress (Wu *et al.* 1996). Xyloglucan endotransglycosylase (XET) has been considered as a cell wall loosening enzyme (Fry *et al.* 1992) and a previous study from Wu *et al.* (1994) has demonstrated stimulated XET activity in the apical 5 mm of maize primary roots at low water potential. Furthermore, Wu *et al.* (2001) have uncovered genetic clues companied with increased cell wall extension in maize root apex under water stress. Up-regulation of the transcript level of four selected expansin genes in root apical region at low water potential is

closely related with enhanced cell-wall extensibility, and expansin activity and abundance. More recently, Barcia *et al.* (2014) has reported that two expansin genes TaEXPB8 and TaEXPA5 were up-regulated in medium water-stressed wheat root. Li AX *et al.* (2015) discovered that root-specific overexpression of a wheat expansin gene TaEXPB23 in transgenic tobacco plant stimulated root growth and enhanced its tolerance to drought. Fewer accumulated reactive oxygen species (ROS) and increased level of antioxidant enzyme activity were observed as well in the transgenic plant.

Saab *et al.* (1990) have shown that elevated levels of endogenous ABA concentration were associated with sustained elongation of primary root in maize (*Zea mays* L.) seedlings at low water potential (-1.6 MPa) and inhibited elongation of shoots at low water potential (-0.3 MPa). Exogenous application of ABA can either stimulate or inhibit primary root growth in maize depending on the initial root elongation rate or the concentration of ABA (Mulkey *et al.* 1983, Pilet and Saugy 1987, Saab *et al.* 1990). In a population of maize primary roots with different elongation rates, white light treatment resulted in decreased growth rate, associated with higher levels of endogenous ABA concentration (Saugy *et al.* 1989). Robertson *et al.* (1985) have reported that endogenous ABA concentration was up-regulated under water stress in the first 3 mm of root apex of sunflower (*Helianthus annaus* L. cv Russian Giant). According to Barlow and Pilet (1984), exogenously applied ABA can have a direct impact on root apical meristem by reducing cell division and DNA synthesis. Robertson *et al.* (1985) assumed that changes in endogenous ABA level in root apex can be a mechanism underlying the regulation of root growth and development under drought. The observation of Robertson *et al.* (1985) is evidently consistent with Sharp *et al.* (1998) who reported that the elongation rate of the first 3 mm of root apex was not influenced under water stress treatment, which indicated that increased ABA content in root apex may account for the maintained growth in root apex.

In our experiment, it is constantly observed in both varieties that root growth under severe water stress where pre-dawn leaf water potentials dropped to as low as -1.0 to -1.5 MPa was remarkably inhibited, as displayed in WS-High section in C and D in Figure 3.3 and Figure 3.5, and WS-High section in B in Figure 3.6 and Figure 3.7. Moreover, based on our observations, when water potentials were too much decreased, the elongation of a large number of roots ceased completely. In addition, visible observations suggested that root diameter was drastically reduced, and more lateral roots were formed especially closer to root apex (data not collected).

Under relatively low level of water stress, changes in root growth seem to be more complicated and less predictable as decreased, unaffected and promoted growth were all recorded. For well-watered plants, $\Psi_{predawn}$ was maintained at a high level (> -0.1 MPa). When plants were under low level of drought treatment, their average $\Psi_{predawn}$ dropped to around -0.2 MPa ~ -0.5 MPa and in most cases, this decrease was not statistically significant. In May 2015, the effect of drought on root growth occurred relatively fast as we can see in B and C in Figure 3.2: root growth of RGM was significantly decreased after 6 days without any irrigation, and root growth of 110R was remarkably reduced after two days without watering. Pre-dawn leaf water potentials were not

measured for this experiment. However, if we refer to the drop of $\Psi_{predawn}$ versus the duration of drought treatment, we can assume that plant water potentials were not significantly affected in 2 or 6 days. One possible explanation for this quick reaction to drought can be ascribed to the certain environment in which the plants were grown. As described in the last section, soil temperature during the experiment in May 2015 was low (see Table 3.2 and Figure 3.13). Furthermore, other environmental factors such as irradiance and air humidity may be accounted for as well. For RGM and 110R from July-August 2015, 110R from May-June 2016, and RGM from July-August 2016, average plant water potentials decreased to around -0.2 MPa ~ -0.3 MPa under low level of water stress but root growth was not influenced. For RGM from May-June 2016, $\Psi_{predawn}$ decreased significantly to around -0.5 MPa and root growth was considerably stimulated during the second half of the low stress level period. For 110R from August-September 2016, $\Psi_{predawn}$ dropped to around -0.5 MPa (not statistically significant) as well, but root growth was already significantly inhibited. From the data we collected, it is clear that in general root growth in grapevine is not affected under low level of drought (e.g., $\Psi_{predawn}$ drops to -0.3 MPa). However, when $\Psi_{predawn}$ continues to decrease, weather root growth rate is modified or not might be genotype-dependent given that RGM and 110R are generally considered to exert very different characteristics in terms of coping with drought stress. Changes in shoot growth during this stage were not determined in our experiments.

3.4.4 Varietal differences

We constantly observed that under well-watered conditions, roots of 110R tended to grow faster than those of RGM, predominantly during the early and late stages within one growing season (e.g., E in Figure 3.2, Figure 3.4, and Figure 3.5). Similarly, under well-watered conditions, root biomass of 110R was significantly higher than that of RGM (panel B in Figure 3.10). However, under water-stressed conditions, the differences in root growth between these two genotypes disappeared and they grew at similar rates and no differences were observed in terms of root system biomass neither. RGM and 110R are commonly recognized with contrasting resistance to drought with RGM being sensitive and 110R being highly tolerant to drought (Carbonneau 1985, Rossdeutsch *et al.* 2016). As suggested by Teskey and Hinekley (1987), increased root system development and root growth benefited white oak's resistance to drought. Therefore, it is not surprising to see the advantage of 110R in root growth rate.

Growth and development of root system is a result of the coordinated control of both endogenous determinant (genetic, regulating growth and organogenesis) and exogenous environmental stimuli (biotic and abiotic) (Malamy 2005, Hodge *et al.* 2009). Even though at present grapevine (*Vitis vinifera* L.) genome has been sequenced and updated since the first available version of Jaillon *et al.* (2007), the two varieties used in our study are not from the same species. Thus, the genetic determinant candidates (if there are any) separating the root growth rate of RGM and 110R remain unrevealed. Nonetheless, hopefully the RNA-seq data we obtained on roots of RGM and 110R (see Chapter 5) can be leveraged in the future to provide more information on these differences.

Phytohormones are well recognized to play important roles in the regulation network of root growth and development, of which auxin is known to be critical for root patterning, primary root growth, lateral root formation and root architecture (Hodge *et al.* 2009, Perrot-Rechenmann 2013). Auxin plays an important role in cell division and cell expansion as well. At present, mechanisms involved in auxin regulation of primary root growth have been attributed to its distribution and concentration as well as intercellular auxin transport (Perrot-Rechenmann 2013).

In addition to auxin, several other phytohormones are also responsible for the regulation of root growth and development, possibly by interacting with auxin activity. Cytokinins have long been known as negative regulators of root growth and development (Perilli *et al.* 2013). Exogenous application of cytokinin suppresses root elongation and lateral root formation (Beemster and Baskin 1998, Hodge *et al.* 2009) and transgenic Arabidopsis with decreased endogenous cytokinin levels favored the growth of primary roots and the formation of lateral roots and produced a larger root system (Werner *et al.* 2003, 2010). In contrast to cytokinins, gibberellins have been recognized as positive regulators of plant growth and development (Tanimoto and Hirano 2013) and they promote root growth and root meristem size by maintaining cell division (Ubeda-Tomás *et al.* 2008, 2009, Achard *et al.* 2009). Exogenous application of gibberellins has been proved to stimulate the size of root meristem (Moubayidin *et al.* 2010). Another phytohormone that has profound influence on plant growth and development is ethylene. Both negative regulation of ethylene on root elongation and lateral root development and stimulatory effects of ethylene on root hair formation have been elucidated (Lewis and Muday 2013). Strong inhibition of cell elongation in elongation zone of the root and resulted short root length have been reported (Le *et al.* 2001). Exogenous ethylene treatment has been shown to inhibit the elongation of the central root elongation zone in Arabidopsis root (Růžička *et al.* 2007, Swarup *et al.* 2007, Strader *et al.* 2010). Moreover, the influence of ethylene on root growth has been demonstrated to be synergistic with another plant hormone, auxin (Růžička *et al.* 2007). Inhibition of root elongation of ethylene by up-regulating auxin biosynthesis in Arabidopsis has been illustrated (Růžička *et al.* 2007, Swarup *et al.* 2007). Abscisic acid (ABA) has been well known as a stress hormone and plays a central role in promoting primary root elongation and lateral root formation in response to water stress (De Smet *et al.* 2006). Generally speaking, ABA is considered as an inhibitor to plant shoot and root growth under both well-watered and water-stressed conditions (Sharp *et al.* 1994, Sharp and LeNoble 2002, Rowe *et al.* 2016). However, the effects of exogenous ABA on root growth under well-watered conditions have shown to be concentration-dependent by some studies (Watts *et al.* 1981, Xu *et al.* 2013). When ABA concentration is relatively low, it promotes root growth, while high ABA concentrations down-regulate root growth. Anyway, the biphasic effects of ABA on root growth regulation is a complex process and might involve synergistic interaction with one or several other plant hormones (Luo *et al.* 2014, Rowe *et al.* 2016, Li *et al.* 2017).

Except for their genetic diversities, differences in root growth between RGM and 110R could be a result of differences in hormonal regulation. For instance, the endogenous concentration of a

certain hormone which is critical for the determination of root elongation rate may be different. Unfortunately, no information is available on the concentration of various hormones in root for these two cultivars. In the study of Rossdeutsch *et al.* (2016), we found that there is no difference in terms of ABA concentration in xylem sap between RGM and 110R. Thus, further investigations are still needed to explore the endogenous differences on the hormone level which might cause different root growth rate in RGM and 110R.

3.5 Concluding remarks

The cylinder rhizotron system tested in our experiment has proved to be a practical and effective method to realize the determination of root growth rate without disturbing the natural growing environment of the root system. Root growth is very plastic and fluctuated tremendously through the period of each experiment for both well-watered and water-stressed plants. In general, especially during the early and late stages within one growing season, 110R grew at a higher speed than RGM under well-watered conditions; however, the differences disappeared under water deficit and roots from both genotypes grew at a similar speed. We assume that higher root growth rate may be one characteristic of 110R which contributes to its higher resistance to drought stress. Water stress treatment has a significant influence on root growth: it reduced root elongation rate on average and shifted the individual root growth distribution curve to the lower speed side. Numerous factors, both endogenous and exogenous, e.g., genes related to root growth and development, level of relative phytohormones, air and soil temperature, light intensity, soil moisture, nutrients availability, soil resistance, etc, can impact root growth and development, and as a result can be responsible for the constantly observed fluctuations in root growth rate. Nowadays, researches in understanding internal determinants in root growth and development and corresponding regulation networks have received great attention, and a lot of progresses have been made in the field concerning plant root system. Nevertheless, there is still a long way to go in order to have a thorough understanding in elements that determine root growth and regulate root responses to various external stimuli.

Chapter 4 Response of root hydraulic conductivity and aquaporin gene expression to water stress

4.1 Introduction

Drought is an increasingly restricting environmental constraint to plant growth and development under the circumstance of a changing climate. It is of great importance to obtain a good understanding of how plants regulate and optimize water uptake when water resource is a limiting factor.

Root hydraulic conductivity is related to plants' ability to absorb water, and it has been shown that root hydraulic conductivity is down-regulated by water stress (e.g., North and Nobel 1996). This down-regulation has been attributed to increased suberization in exodermis and endodermis of the root tip in the aspect of changes in anatomical structure (e.g., Barrios-Masias *et al.* 2015). At the molecular level, aquaporins have been recognized to participate in the regulation of root water uptake. However, changes in the expression level of various aquaporin genes under water-stressed conditions have been very dynamic and no consistent trend has been defined.

The two varieties studied in our experiment are known to have contrasting capacities in drought resistance. RGM is sensitive to drought stress while 110R is tolerant to drought stress. The aims of the experiments described in this chapter were to investigate the responses of root hydraulic conductivity to water stress as well as changes in the expression level of some aquaporin genes selected, and to integrate these changes with the changes in root growth described in the previous chapter.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

Root samples analyzed in this chapter were from the experiments conducted in the 2016 growing season, which were RGM and 110R from May-June 2016, RGM from July-August 2016, and 110R from August-September 2016. Measurement of root hydraulic conductivity was performed on the RGM and 110R plants from May-June and on the 110R plants from August-September. mRNA extraction and qPCR analysis were carried out on the RGM plants from July-August 2016 and the 110R plants from August-September. With regard to plant materials and growth conditions as well as methods of measurement of root growth and water potentials, they are identical as described in chapter 3.

4.2.2 Measurement of root hydraulic conductivity (Lp_r)

Hydraulic conductivity of individual roots with known growth rate was determined using an osmotic pressure gradient with a meniscus tracking method. Root sampling took place between 10h00 and 12h00. Targeted roots were marked one by one before sampling in order to be distinguished from each other. Growing medium around one targeted root was carefully removed, and the root was maintained intact. A ticket made from adhesive tape was sticked around this root. After all targeted roots were labeled, the whole grapevine was carefully removed from the rhizotron and the whole root system was submerged in water. Then the plant was brought back to the laboratory immediately. Labeled roots were cut off with a razor blade under water and glued into a 500-mm-diameter glass capillary via a home-made adaptor. When cutting off the single roots for Lp_r measurement, they were kept as long as possible in terms of length and lateral roots were avoided as much as possible. The capillary was filled with deionized water (diH_2O) and the water-air interface was observed with a webcam as a meniscus. The webcam was connected to a laptop and YAWCAM (Version 0.5.0) was used to take pictures of the capillary every 30 seconds in order to calculate the movement of the meniscus and to further obtain the hydraulic conductance of the root portion. Figure 4.1A displays the setup of the measurement and 4.1B presents an example of the calculation of Lp_r across a range of pressures for both control and inhibited conditions. ImageJ (1.51a, Wayne Rasband) was used to calculate the pictures in order to have the speed of the movement of the meniscus. Then the flow rate can be obtained based on the speed of movement of the meniscus. Sucrose solutions of different concentrations were made to create different osmotic pressures. All solutions were aerated during the measurement. The relationship between the osmotic pressure of a sucrose solution and its concentration is established by the osmosis equation:

$$\pi = iMRT$$

where,

π is the osmotic pressure in ATM;

i is the van't Hoff factor of the solute, for sucrose the van't Hoff factor is 1 as it does not dissociate in water;

M is the molar concentration in mol/L;

R is the universal gas constant which equals to 0.08206 L·atm/mol·K;

T is the absolute temperature in K.

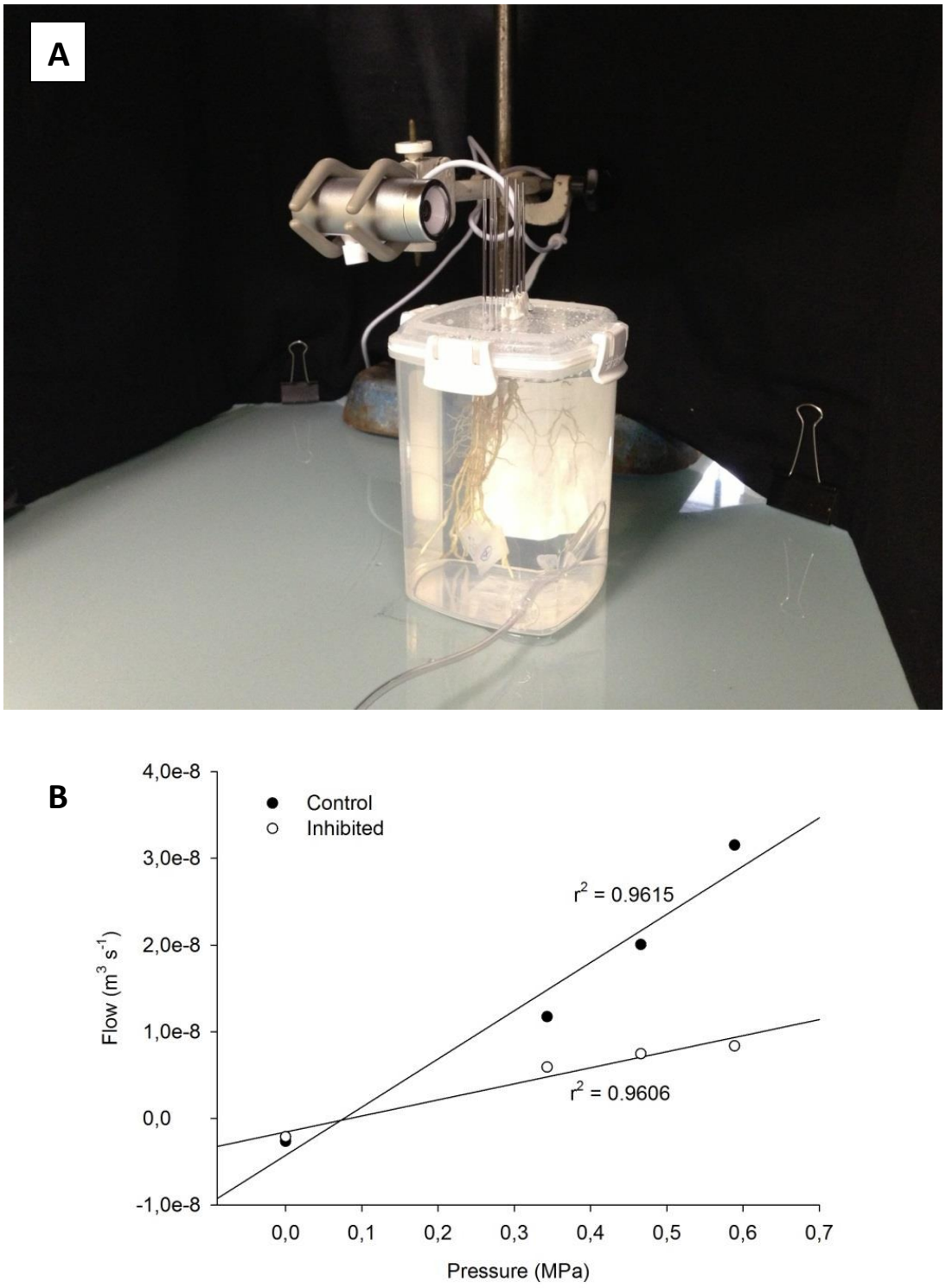

Figure 4.1 Experiment setup (A) and examples of calculation (B) for Lp_r measurement. Examples of pressure-flow relationship for the calculation of Lp_r for both control (black circles) and 1 mM NaN_3 inhibited (empty circles) conditions are presented in panel B.

Then the relationship between flow rate and osmotic pressure is plotted to calculate the hydraulic conductance of the root (as in Figure 4.1B). The length and diameter of each root were measured in order to estimate the root surface area. Lp_r was finally calculated by normalizing hydraulic conductance by root surface area. After the determination of Lp_r, the measurement was repeated by using 1mM sodium azide (NaN_3) as a chemical inhibitor to aquaporins under the same osmotic pressures. An inhibited Lp_r value was then obtained.

4.2.3 Total RNA extraction and real-time quantitative PCR (RT-qPCR)

Root tips of 5 cm long with known growth rate were harvested and frozen immediately in liquid nitrogen and kept in a -80 °C refrigerator until the time of analysis. Frozen samples were ground under liquid nitrogen in 2-ml eppendorf tubes with a small plastic pestle into powder for RNA extraction. Total mRNA was extracted after Reid *et al.* (2006). Genomic DNA contamination was removed with the Turbo DNA-free kit (Life technologies, according to the manufacturer's instructions) and reverse transcription was performed using iScript advanced cDNA synthesis kit (Bio-Rad) with oligo dT primers and 1.0-1.5 µg of RNA according to the manufacturer's instructions. Transcript abundance of *Vv*PIP1,1, *Vv*PIP 1,2,4, *Vv*PIP 1,3,5, *Vv*PIP 2,1, *Vv*PIP 2,2, *Vv*PIP 2,3, and *Vv*PIP 2,4 was analyzed on a Bio-Rad CFX96 machine using iQ SYBR Green Supermix (according to the manufacturer's instructions). The transcript abundance level of the selected genes was normalized to the geometric mean of *Vv*GAPDH, *Vv*EF1 and *Vv*Actin expression (Reid *et al*. 2006). The relative gene expression level was calculated according to the 2-ΔΔCT method (Livak and Schmittgen 2001). The primers used for RT-qPCR have been designed by Gambetta *et al.* (2012) and the sequences are presented in Table 4.1.

Table 4.1 Sequences of the primers used for RT-qPCR (Gambetta *et al.* 2012)

	Forward primer (5' to 3')	Reverse primer (5' to 3')
*Vv*PIP1,1	GAGTGGTGCTGGGCGTTGATC	GTGGAATGCTACAGACATTAC
*Vv*PIP1,2,4	GTTTCTTCTTTTATTTGCTGC	GCTGCCCATTGTAATAGAAGC
*Vv*PIP1,3,5	CCATTCAAGAGCAGGGCTTGAG	ATTTACACACTTAGGTAGTAGG
*Vv*PIP2,1	CCATTTTGATACCTTCTTCC	TATCTACAATTTCATGCCCTC
*Vv*PIP2,2	AACTAAAAACCCACAACACCC	CATCATCATAATCATCTCTGG
*Vv*PIP2,3	CATTTCAATCCACATGGTCCG	CCACAAATTCGTCACACATCC
*Vv*PIP2,4	GATGACCATTGGATGTTCTGG	GCTTTAATGGCCGCTGCTCTC
*Vv*Actin	CTTGCATCCCTCAGCACCTT	TCAATCTGTCTAGGAAAGGAAG
*Vv*EF1	CAAGAGAAACAATCCCTAGCTG	TCAATCTGTCTAGGAAAGGAAG
*Vv*GAPDH	CCACAGACTTCATCGGTGACA	TTCTCGTTGAGGGCTATTCCA

4.2.4 Statistical analysis

The effects of drought treatment and developmental stages on pre-dawn leaf water potential, hydraulic conductivity, as well as aquaporin expression were analyzed using a two-way ANOVA ($p < 0.05$, with Tukey's HSD test). When the data did not meet normal distribution, the Kruskall-Wallis test was run on each factor and then pairwise Wilcoxon test was used to separate the difference ($p < 0.05$). The relationship between relative gene expression level and $\Psi_{predawn}$, between individual root hydraulic conductivity and root growth rate, as well as between relative gene expression level and root growth rate, was examined using the generalized linear model (GLM), and when necessary ($p < 0.05$), a linear regression line was fitted on the scattered graph using SigmaPlot (Version 11.0, Systat Software). All ANOVA and GLM analyses were realized in R version 3.3.1 (2016-06-21) (R Core Team) and all graphs were created with SigmaPlot (Version 11.0, Systat Software).

4.3 Results

4.3.1 Root hydraulic conductivity

Individual root hydraulic conductivity (Lp_r) was determined for both genotypes under well-watered and water-stressed conditions. A summary of the results from two-way ANOVA on the effect of treatment and developmental stage on $\Psi_{predawn}$, Lp_r, inhibited Lp_r, as well as the percentage drop of Lp_r after inhibition was illustrated for RGM and 110R in Table 4.2 and 4.3, respectively.

During the period of each experiment, three stages, early, mid, and late, were defined according to the time scale (as in chapter 3). Pre-dawn water potentials of water-stressed plants dropped significantly with prolonged drought treatment, while those of well-watered plants were maintained at a high level through the period of the experiment (insets in A in Figure 4.2, 4.3, and 4.4).

Quantification of individual root hydraulic conductivity revealed significant differences between different treatment and developmental changes. In RGM, stress treatment resulted in significant decreases in Lp_r with Lp_r CT being two times greater than Lp_r WS during the early stage when $\Psi_{predawn}$ dropped slightly due to drought treatment (A in Figure 4.2 and inset in A). Stress treatment did not significantly affect Lp_r in mid and late developmental stages. Interestingly, even under well-watered conditions, Lp_r reduced significantly in mid and late stages in comparison to early stage with an approximately 66% decrease (A in Figure 4.2). When aquaporin activity was inhibited by 1 mM NaN_3, no differences in Lp_r were observed neither between treatments nor between different stages (A in Figure 4.2). The percentage reduction in Lp_r under inhibition was about 40% to 80% and was significantly lower in stressed plants in late stage compared with the other conditions (B in Figure 4.2). In 110R, changes in Lp_r in response to drought treatment and developmental changes from the two experiments conducted were consistent with RGM. Water deficit resulted in reductions in Lp_r, non-significant in the May-June experiment (30% drop in

average, A in Figure 4.3) and significant in the August-September experiment (60% drop in average, A in Figure 4.4), in early stage when $\Psi_{predawn}$ was decreased slightly from drought treatment (insets in A in Figure 4.3 and 4.4), while no variances were found in mid and late stages. Moreover, stress treatment did not have a significant impact on Lp_r in mid and late stages (A in Figure 4.3 and 4.4). Again, under well-watered conditions, Lp_r decreased significantly in mid and late stages in contrast to early stage with an approximately 75% drop in May-June (A in Figure 4.3) and 60% drop in August-September (A in Figure 4.4). Inhibited Lp_r value of early stage was not available in the May-June experiment. Nonetheless, with inhibition, no differences were observed neither between treatments in mid and late stages nor between these two stages. Inhibited Lp_r value from early stage under non-stressed conditions was higher than the other conditions (A in Figure 4.3). A 50% to 95% percentage drop of Lp_r under inhibition was observed in 110R in the May-June experiment. However, due to large variances within certain conditions (e.g., WS in late stage), no significant effects of treatment or develpmental stage were observed (B in Figure 4.3). In the August-September experiment carried out in 110R, with inhibition, a significant effect of treatment on Lp_r was found in early and mid stages with Lp_r CT being approximately two folds greater. Across three stages, Lp_r CT was obviously higher in early and mid stages than in late stage while no differences were found for Lp_r WS (A in Figure 4.4). A 60% to 85% percentage drop in Lp_r under inhibited condition was observed in 110R in the August-September experiment. Within each stage, treatment did not influence the percentage drop with inhibition. However, the percentage drop in mid stage for both CT and WS was found to be lower than the other stages (B in Figure 4.4). Additionally, Lp_r of all individual roots measured in our experiment was plotted against $\Psi_{predawn}$ to illustrate changes in Lp_r in response to the level of $\Psi_{predawn}$ (Figure 4.5). On an individual fine root level, Lp_r showed a fast drop in the beginning of the water stress treatment when $\Psi_{predawn}$ was higher than -0.5 MPa, and in the results we obtained, this drop was more pronounced in RGM than in 110R. With $\Psi_{predawn}$ getting more negative, e.g. from -0.4 MPa to -2.0 MPa, the range of Lp_r values measured in our experiment was maintained constant. Lp_r of well-watered plants decreased as well even though their $\Psi_{predawn}$ was maintained at a high level (< 0.1 MPa) during the period of the experiment.

Table 4.2 Summary of results from two-way ANOVA on the effect of treatment and developmental stage on $\Psi_{predawn}$, Lp_r, inhibited Lp_r, and the percentage drop of Lp_r after inhibition for RGM from the May-June 2016 experiment.

	RGM May-June		
	Treatment	**Stage**	**Treatment x Stage**
$\Psi_{predawn}$	***	**	*
Lp_r	**	***	*
Lp_r inhibited	ns	ns	ns
Perecntage drop of Lp_r	ns	*	ns

Significant codes: *** 0.001, ** 0.01, * 0.05, ns not significant

Table 4.3 Summary of results from two-way ANOVA on the effect of treatment and developmental stage on $\Psi_{predawn}$, Lp_r, inhibited Lp_r, and the percentage drop of Lp_r after inhibition for 110R from the May-June 2016 and the August-September 2016 experiment.

	110R May-June			110R August-September		
	Treatment	**Stage**	**Treatment x Stage**	**Treatment**	**Stage**	**Treatment x Stage**
$\Psi_{predawn}$	***	***	***	***	***	***
Lp_r	ns	***	ns	***	***	*
Lp_r inhibited	ns	**	ns	***	***	ns
Perecntage drop of Lpr	ns	ns	ns	ns	***	ns

Significant codes: *** 0.001, ** 0.01, * 0.05, ns not significant

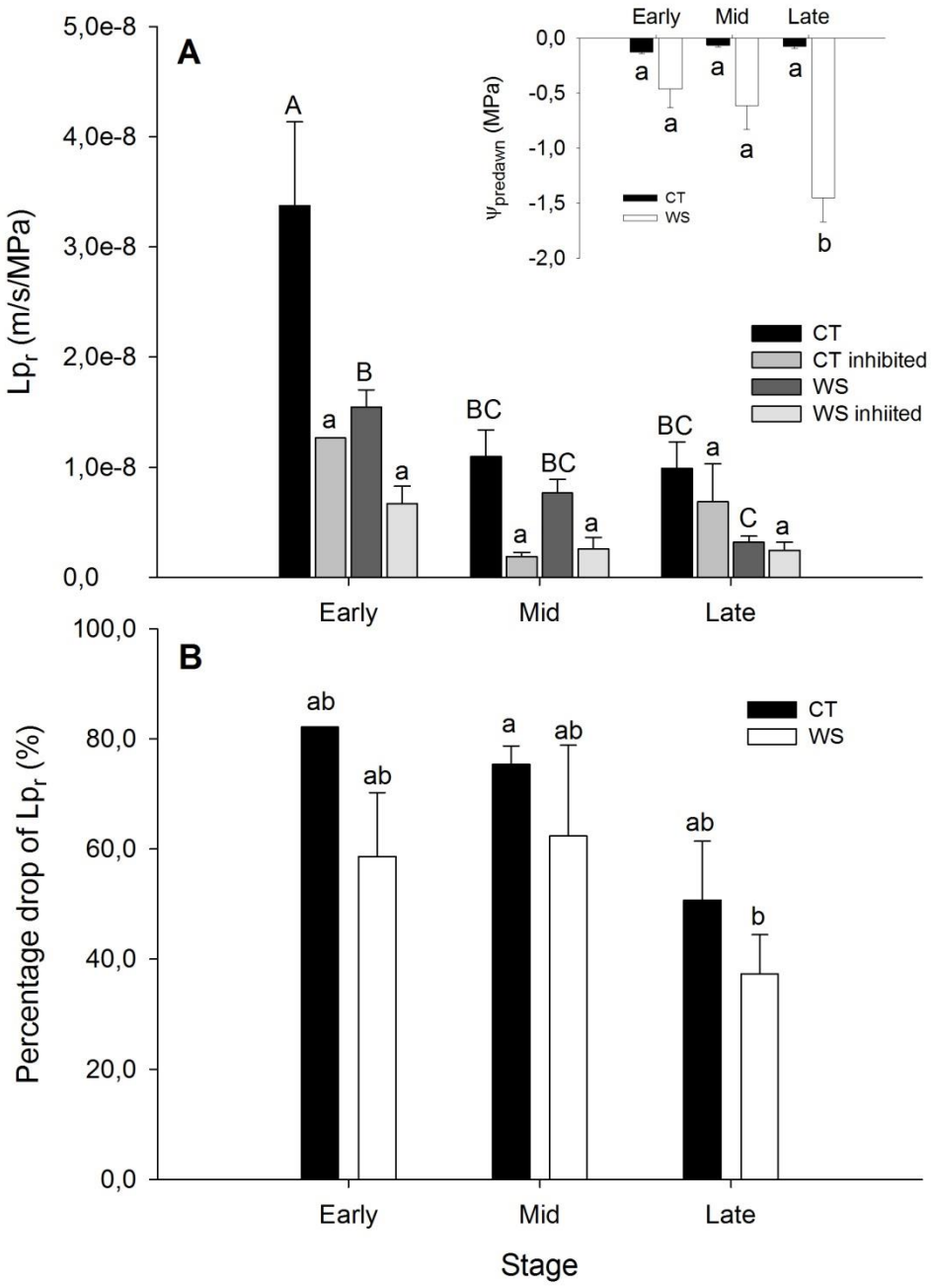

Figure 4.2 Hydraulic conductivity Lp_r and aquaporin inhibition in grapevine fine roots, RGM May-June 2016. A, Lp_r of individual fine roots from both well-watered and water-stressed plants across three developmental stages obtained under an osmotic pressure (black and dark grey bars under capital letters). Inhibited Lp_r values were plotted next to non-inhibited ones (grey and light grey bars under small letters). Different capital letters represent values that are significantly different under non-inhibited condition (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 8, n-WS-Early = 24, n-CT-Mid = 10, n-WS-Mid = 14, n-CT-Late = 7, n-WS-Late = 14. Under inhibited condition, no significant differences were

detected between treatment and developmental stages (Kruskall-Wallis test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 1, n-WS-Early = 4, n-CT-Mid = 8, n-WS-Mid = 5, n-CT-Late = 4, n-WS-Late = 6. Inset in A is $\Psi_{predawn}$ for both CT and WS across three stages and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of plants for each condition is: n-CT-Early = 2, n-WS-Early = 5, n-CT-Mid = 3, n-WS-Mid = 3, n-CT-Late = 4, n-WS-Late = 5. B, Percentage of reduction in Lp_r when aquaporins were inhibited with 1 mM NaN_3 for both CT and WS across three stages. Different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-CT-Early = 1, n-WS-Early = 4, n-CT-Mid = 8, n-WS-Mid = 5, n-CT-Late = 4, n-WS-Late = 6). All values are mean ± SE.

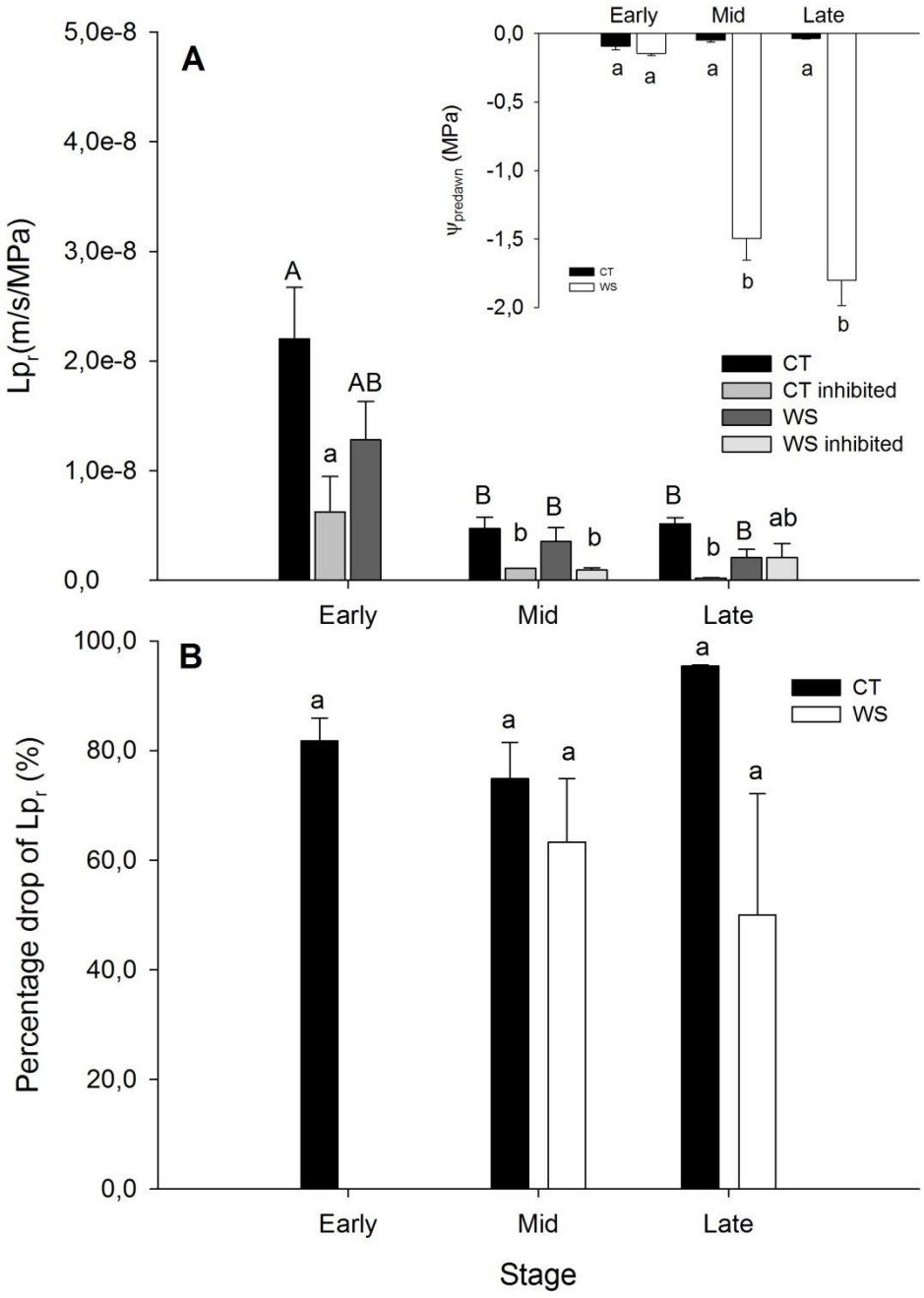

Figure 4.3 Hydraulic conductivity Lp_r and aquaporin inhibition in grapevine fine roots, 110R May-June 2016. A, Lp_r of individual fine roots from both well-watered and water-stressed plants across three developmental stages obtained under an osmotic pressure (black and dark grey bars under capital letters). Inhibited Lp_r values were plotted next to non-inhibited ones (grey and light grey bars under small letters). Different capital letters represent values that are significantly different under non-inhibited condition (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 7, n-WS-Early = 10, n-CT-Mid = 3, n-WS-Mid = 5, n-CT-Late = 10, n-WS-Late = 6. Small letters represent values that are significantly different

under inhibited condition (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 2, n-WS-Early = 0, n-CT-Mid = 3, n-WS-Mid = 5, n-CT-Late = 2, n-WS-Late = 3. Inset in A is $\Psi_{predawn}$ for both CT and WS across three stages and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of plants for each condition is: n-CT-Early = 2, n-WS-Early = 2, n-CT-Mid = 2, n-WS-Mid = 2, n-CT-Late = 3, n-WS-Late = 3. B, Percentage of reduction in Lp_r when aquaporins were inhibited with 1 mM NaN_3 for both CT and WS across three stages. Different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-CT-Early = 2, n-WS-Early = 0, n-CT-Mid = 3, n-WS-Mid = 5, n-CT-Late = 2, n-WS-Late = 3). All values are mean ± SE.

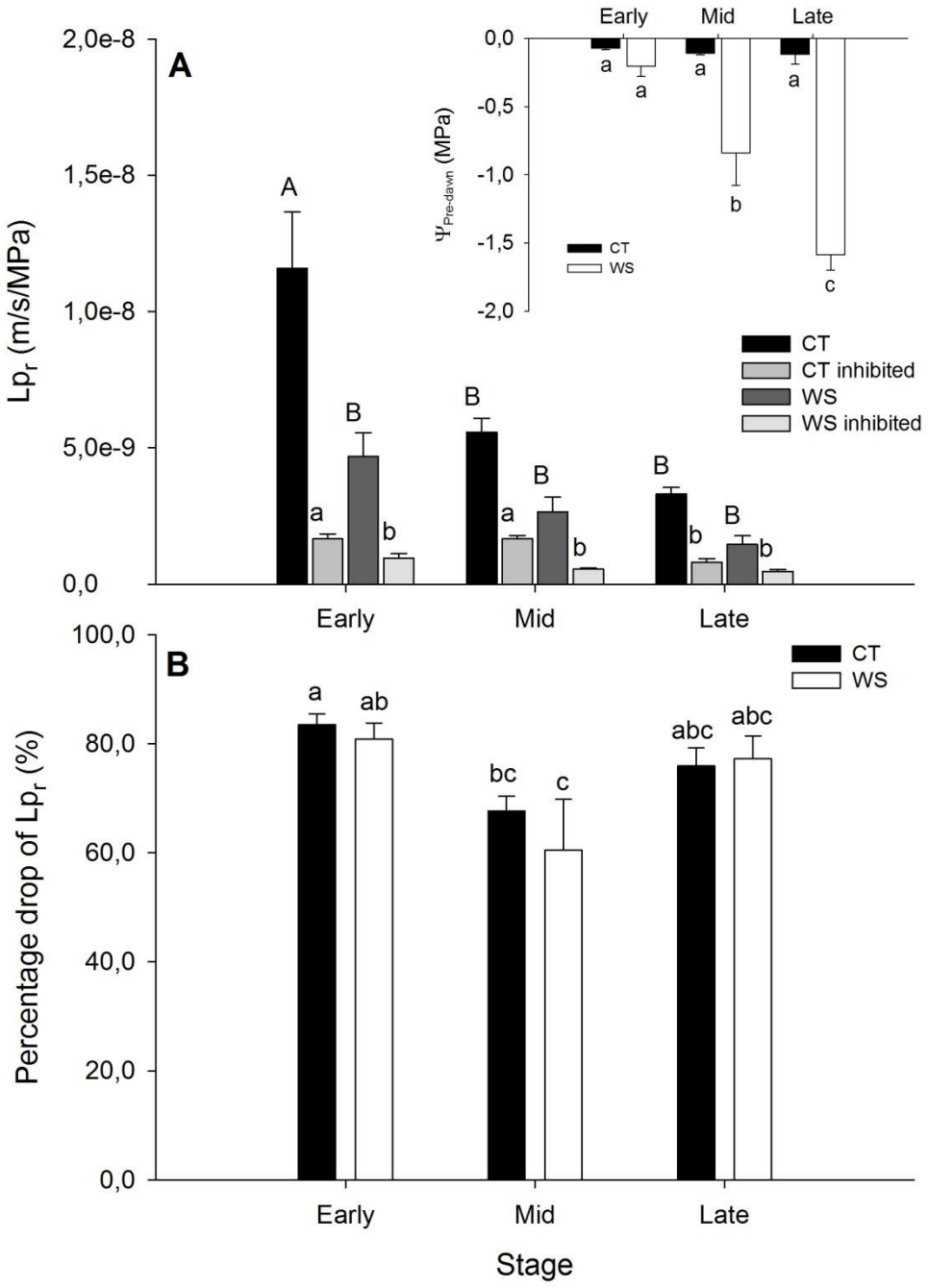

Figure 4.4 Hydraulic conductivity Lp_r and aquaporin inhibition in grapevine fine roots, 110R August-September 2016. A, Lp_r of individual fine roots from both well-watered and water-stressed plants across three developmental stages obtained under an osmotic pressure (black and dark grey bars under capital letters). Inhibited Lp_r values were plotted next to non-inhibited ones (grey and light grey bars under small letters). Different capital letters represent values that are significantly different under non-inhibited condition (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 14, n-WS-Early = 9, n-CT-Mid =

16, n-WS-Mid = 12, n-CT-Late = 15, n-WS-Late = 7. Small letters represent values that are significantly different under inhibited condition (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Number of individual roots measured is: n-CT-Early = 14, n-WS-Early = 7, n-CT-Mid = 16, n-WS-Mid = 5, n-CT-Late = 15, n-WS-Late = 6. Inset in A is $\Psi_{predawn}$ for both CT and WS through three stages and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$. n=3). B, Percentage of reduction in Lp_r when aquaporins were inhibited with 1 mM NaN_3 for both CT and WS across three stages. Different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-CT-Early = 14, n-WS-Early = 7, n-CT-Mid = 16, n-WS-Mid = 5, n-CT-Late = 15, n-WS-Late = 6). All values are mean ± SE.

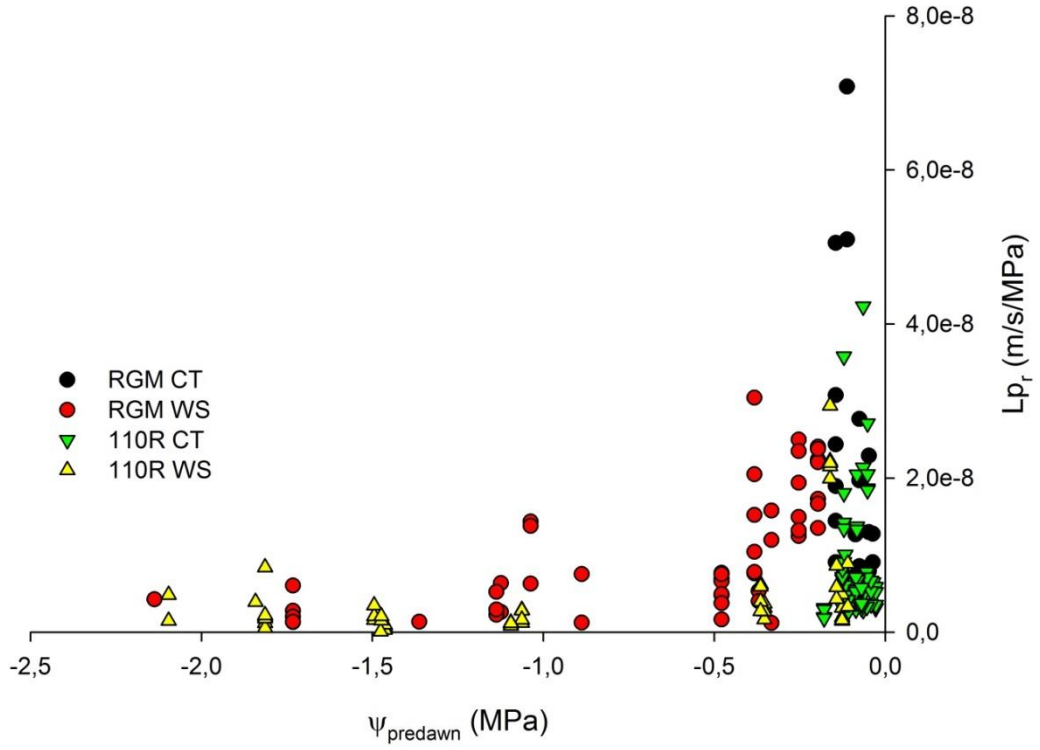

Figure 4.5 Individual root hydraulic conductivity plotted against pre-dawn leaf water potential. Values are raw data of the two varieties from all the individual roots measured in two experiments (the May-June and August-September experiment).

4.3.2 Changes in the expression of aquaporin genes

The expression level of 7 aquaporin genes, *Vv*PIP1,1, *Vv*PIP1,2,4, *Vv*PIP1,3,5, *Vv*PIP2,1, *Vv*PIP2,2, *Vv*PIP2,3, and *Vv*PIP2,4, in response to water stress treatment and developmental stages were studied for RGM from the July-August 2016 experiment and for 110R from the August-September 2016 experiment. As mentioned before, during the period of each experiment, three stages, early, mid, and late, were categorized according to the time scale. $\Psi_{predawn}$ of RGM dropped from around -0.15 MPa to -0.85 MPa under water stress treatment, while $\Psi_{predawn}$ of well-watered RGM was kept at higher than -0.07 MPa (A in Figure 4.6). $\Psi_{predawn}$ of 110R decreased from around -0.2 MPa to -1.6 MPa under water stress treatment, while $\Psi_{predawn}$ of well-watered 110R was maintained at around -0.1 MPa (B in Figure 4.6).

3 isogenes from the PIP1 family and 4 iosgenes from the PIP2 family were studied in our experiment. A and C from Figure 4.7 to Figure 4.13 illustrated the relative expression level of aquaporin genes on average from each developmental stage under well-watered and water-stressed conditions for RGM and 110R, respectively. Ideally, the expectation of water stress treatment was to have $\Psi_{predawn}$ keep decreasing with time going on after irrigation was stopped for the purpose of drought treatment. Nevertheless, in practice, the drying-down process was not always homogenous between plants. As a result, the degree of water stress does not correspond exactly to the evolution of developmental stages. Therefore, in the meantime, the aquaporin gene expression level of each individual root in relation to their $\Psi_{predawn}$ was also analysed and presented in B and D from Figure 4.7 to Figure 4.13 for RGM and 110R, respectively.

4.3.2.1 Response of aquaporin gene expression to water stress and developmental stages

*Vv*PIP1,1 was the most abundantly expressed aquaporin gene among the 7 genes analysed for both varieties, and similar expression levels between these two varieties were observed. The expression of *Vv*PIP1,1 was up-regulated under water stress in RGM in early stage but no differences were observed in mid and late stages between CT and WS; developmental stages did not influence the expression of *Vv*PIP1,1 in RGM (A in Figure 4.7). Nonetheless, *Vv*PIP1,1 expression in 110R was not affected by drought treatment regardless of the developmental changes; similarly, stages did not affect the expression of *Vv*PIP1,1 in 110R (C in Figure 4.7).

A significant up-regulation of *Vv*PIP1,2,4 caused by water stress treatment was seen in RGM in early and late stages, but not in the mid stage, and developmental stage was not an impact factor for the expression of *Vv*PIP1,2,4 in RGM (A in Figure 4.8). Drought treatment did not modify the expression of *Vv*PIP1,2,4 in 110R across all three stages. However, the expression abundance of *Vv*PIP1,2,4 in 110R declined when plants reached at late developmental stage, and this is the case for both CT and WS, which means only developmental stages had an impact on the changes of *Vv*PIP1,2,4 expression (C in Figure 4.8). The expression levels of *Vv*PIP1,2,4 in RGM and 110R were of the same magnitude.

The expression of *Vv*PIP1,3,5 showed an up-regulation in RGM under water stress in early stage. In mid and late stages, water deficit did not produce any significant differences in the expression of *Vv*PIP1,3,5 in RGM (A in Figure 4.9). In 110R, *Vv*PIP1,3,5 expression increased in mid stage in response to water stress (C in Figure 4.9). For both RGM and 110R, *Vv*PIP1,3,5 was the least expressed PIP1 gene among these 3 genes. However, the expression magnitude was at least 10 times higher in RGM than in 110R.

The expression of *Vv*PIP2,1 was up-regulated in RGM under water stress only in early stage, and there was no difference between the expression level of different stages (A in Figure 4.10). The expression of *Vv*PIP2,1 was significantly up-regulated under water stress in early and mid stages in 110R, while in late stage no difference was observed between CT and WS. The level of the expression of *Vv*PIP2,1 maintained stable in CT across three developmental stages (C in Figure 4.10). However, the expression magnitude of *Vv*PIP2,1 was 20 times higher in 110R than in RGM.

The expression of *Vv*PIP2,2 in RGM did not vary between CT and WS through all three stages. However, developmental changes impacted the expression abundance of *Vv*PIP2,2 in RGM. To be more specific, with the evolution in developmental stages, for well-watered plants, the expression of *Vv*PIP2,2 showed a decreased and then increased trend, and for water-stressed plants, a decreased trend (A in Figure 4.11). *Vv*PIP2,2 expression in 110R was up-regulated under water stress treatment in early stage and the expression level decreased in late stage for both water conditions (C in Figure 4.11). The expression of *Vv*PIP2,2 between RGM and 110R was comparable. *Vv*PIP2,2 was the most highly expressed PIP2 gene in 110R.

The expression of *Vv*PIP2,3 significantly increased under drought treatment in early and late stages for RGM. Developmental stage did not alter the expression level of *Vv*PIP2,3 in RGM (A in Figure 4.12). *Vv*PIP2,3 expression was remarkably up-regulated under water stress in 110R in early and mid stages but not in late stage. Therefore, compared with early and mid stages, *Vv*PIP2,3 expression under water stress in late stage was significantly reduced. On the contrary, *Vv*PIP2,3 expression in CT did not differ between stages (C in Figure 4.12). Similar expression level in RGM and 110R was observed.

Drought treatment did not result in any differences in the expression level of *Vv*PIP2,4 in both RGM and 110R (A and C in Figure 4.13). For RGM, no significant variation in well-watered plants across developmental stages was observed, but under stressed conditions, a reduction in the expression of *Vv*PIP2,4 was noticed in mid and late stages. Only developmental stage had an impact on the expression of *Vv*PIP2,4 in 110R with a decreased level in late stage for both CT and WS. However, the expression magnitude of *Vv*PIP2,4 in RGM was 5 times higher than in 110R. *Vv*PIP2,4 and *Vv*PIP2,2 were two most abundantly expressed PIP2 genes in RGM, and *Vv*PIP2,4 was the second most highly expressed PIP2 gene in 110R.

4.3.2.2 Relationship between aquaporin gene expression and $\Psi_{predawn}$

Relative expression level of each aquaporin gene of all individual roots across the period of each experiment was plotted versus $\Psi_{predawn}$ of the plant from which the root was sampled (B and D from Figure 4.7 to 4.13). For both RGM and 110R, the relationship between $\Psi_{predawn}$ and relative gene expression level under water-stressed condition was analyzed using the generalized linear model (GLM). Under the circumstances where the relationship was significant, a linear regression line was fitted and the value of r^2 was presented on the graph. The analysis showed that in our experiment, the expression of some aquaporin genes under drought treatment were not related to the level of $\Psi_{predawn}$, which indicated that the expression of these aquaporin genes was not affected by the degree of water stress. These genes include *Vv*PIP1,1 and *Vv*PIP1,2,4 in RGM, and *Vv*PIP1,3,5 in 110R. For the rest of the aquaporin genes studied, a significant correlation was found under drought treatment between the expression abundance and the value of $\Psi_{predawn}$. More specifically, the expression of aquaporin genes showed a decreased trend with water stress getting more severe.

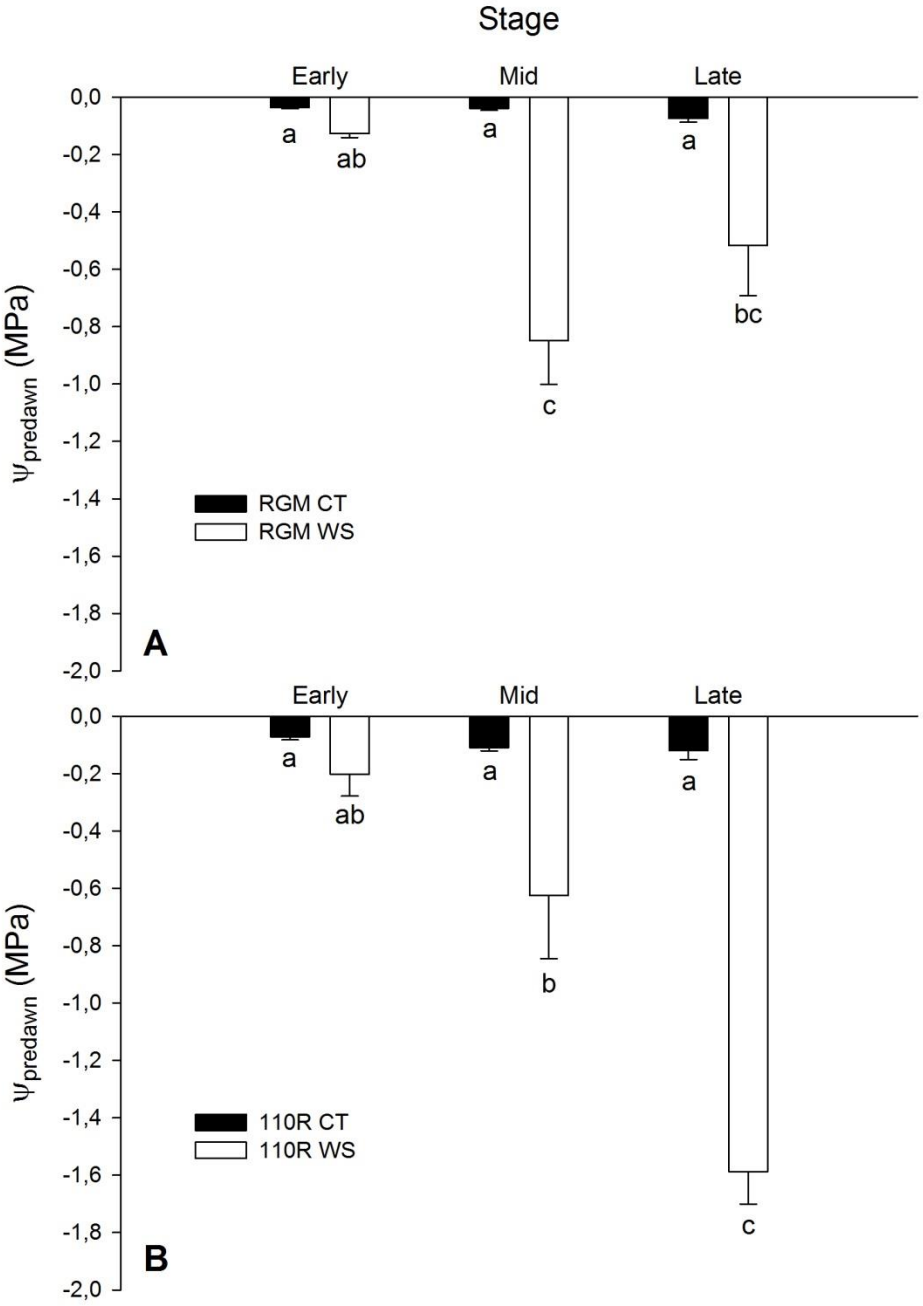

Figure 4.6 Pre-dawn leaf water potential of well-watered and water-stressed plants across three stages. A, RGM from the July-August 2016 experiment. B, 110R from the August-September 2016 experiment. All values are mean ± SE. Different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM = 5-6, n-110R = 3).

Table 4.4 Summary of results from two-way ANOVA on the effect of treatment and developmental stage on $\Psi_{predawn}$, and expression of seven aquaporin genes for RGM from the July-August 2016 experiment and 110R from the August-September 2016 experiment.

	RGM			110R		
	Treatment	**Stage**	**Treatment x Stage**	**Treatment**	**Stage**	**Treatment x Stage**
$\Psi_{predawn}$	***	**	**	***	***	***
***Vv*PIP1.1**	*	ns	**	ns	ns	ns
***Vv*PIP1.2.4**	***	ns	**	ns	***	ns
***Vv*PIP1.3.5**	***	**	**	**	**	ns
***Vv*PIP2.1**	*	ns	*	***	***	**
***Vv*PIP2.2**	ns	***	*	***	***	ns
***Vv*PIP2.3**	***	ns	***	***	**	**
***Vv*PIP2.4**	ns	**	*	ns	***	ns

Significant codes: *** 0.001, ** 0.01, * 0.05, ns not significant

Table 4.5 Summary of results of the relationship between pre-dawn leaf water potential and aquaporin gene expression from generalized linear model for water-stressed RGM and 110R plants from the July-August 2016 experiment and the August-September 2016 experiment, respectively.

	RGM WS	**110R WS**
***Vv*PIP1.1**	ns	*
***Vv*PIP1.2.4**	ns	***
***Vv*PIP1.3.5**	*	ns
***Vv*PIP2.1**	*	***
***Vv*PIP2.2**	**	***
***Vv*PIP2.3**	*	***
***Vv*PIP2.4**	**	**

Significant codes: *** 0.001, ** 0.01, * 0.05, ns not significant

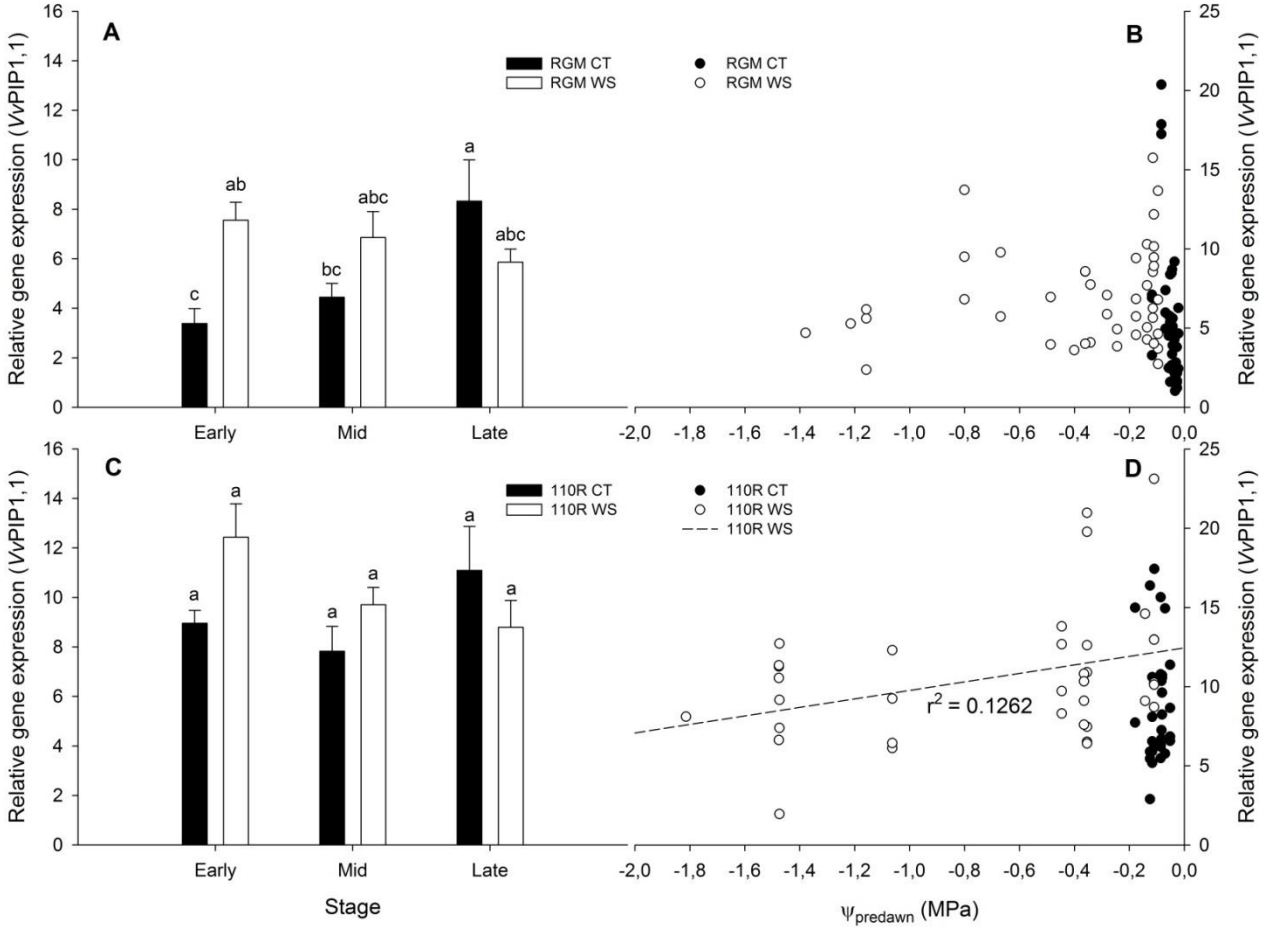

Figure 4.7 Average relative gene expression level of *Vv*PIP1,1 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP1,1 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP1,1 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE, and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP1,1 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

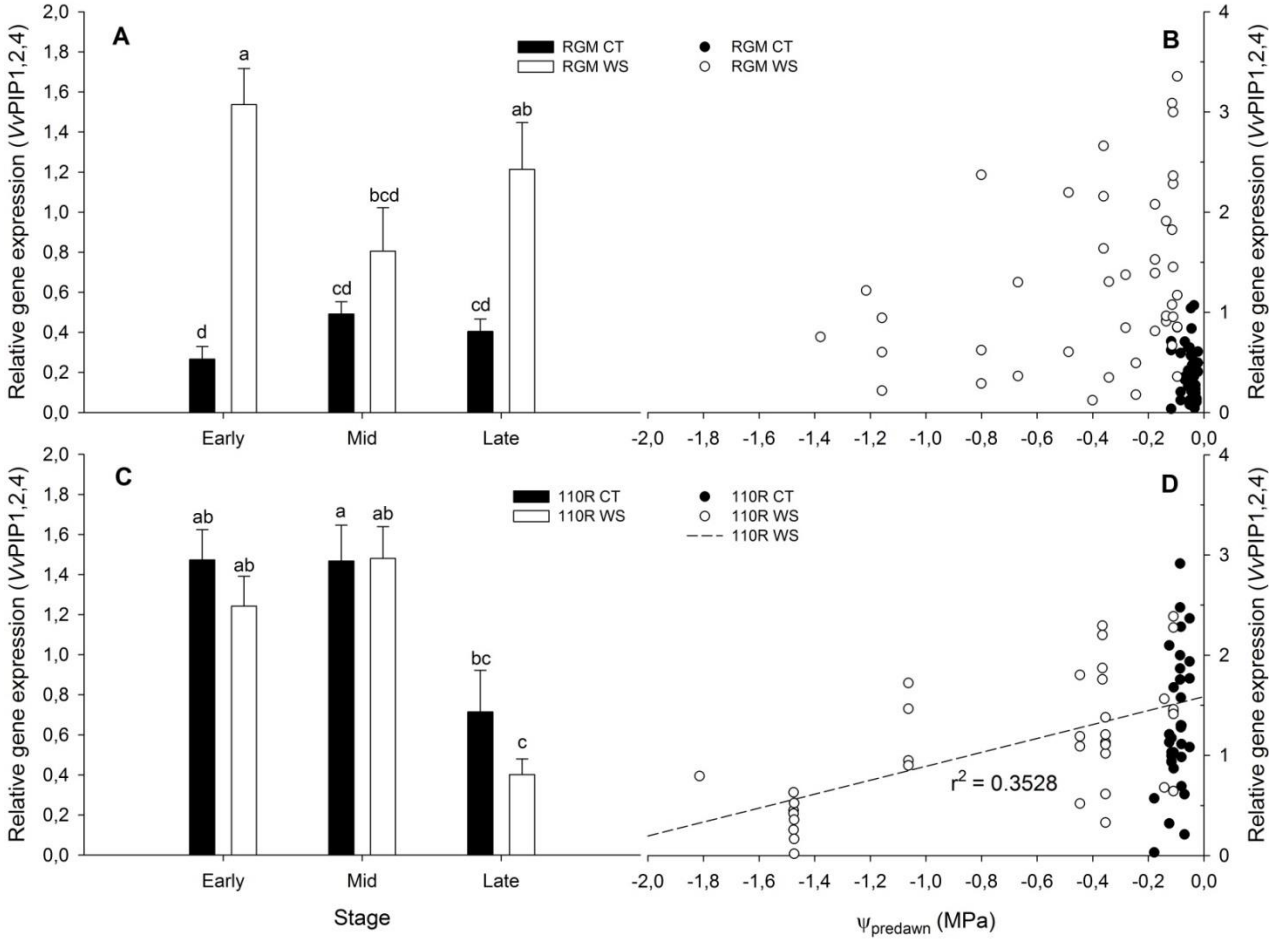

Figure 4.8 Average relative gene expression level of *Vv*PIP1,2,4 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP1,2,4 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP1,2,4 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE, and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP1,2,4 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

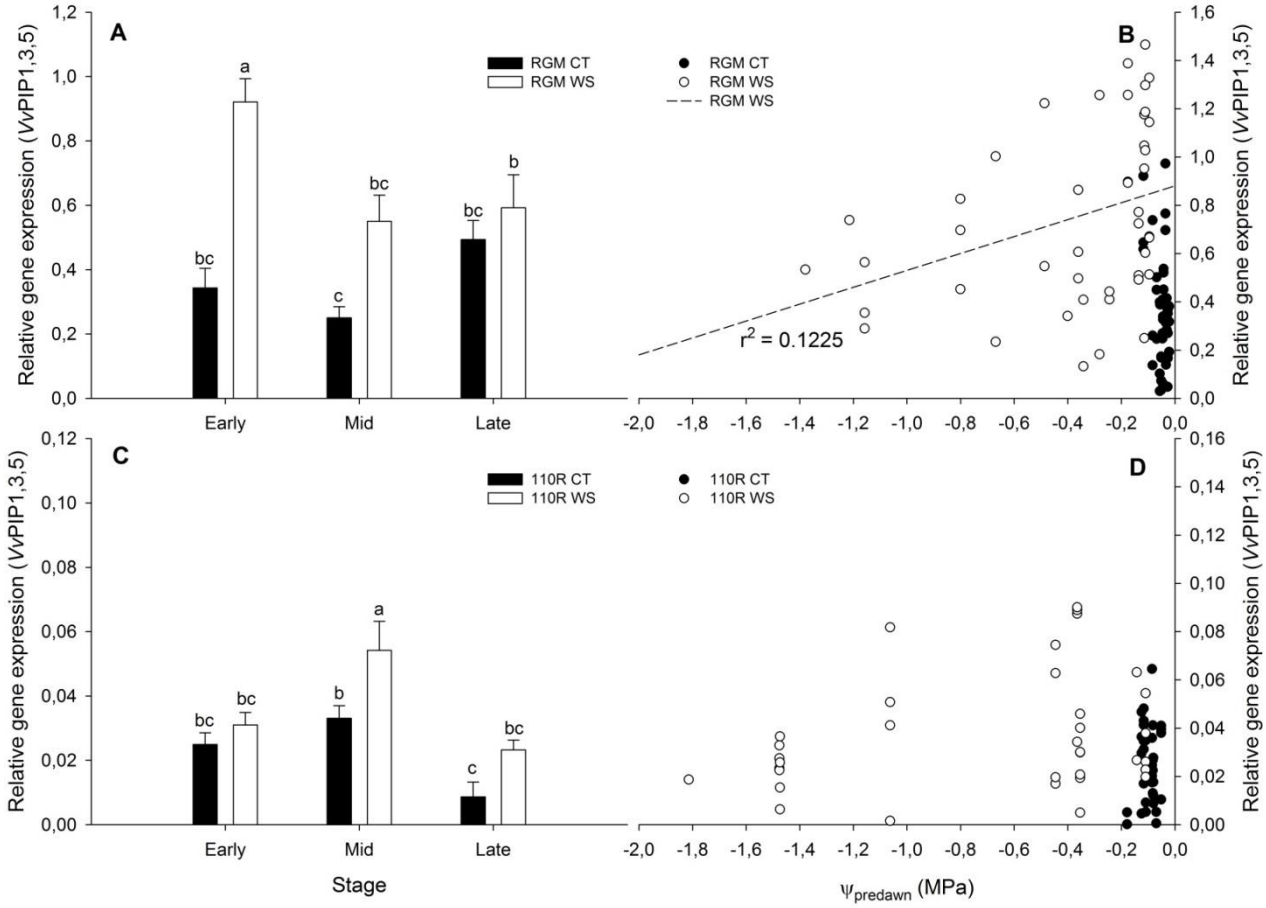

Figure 4.9 Average relative gene expression level of *Vv*PIP1,3,5 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP1,3,5 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP1,3,5 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP1,3,5 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

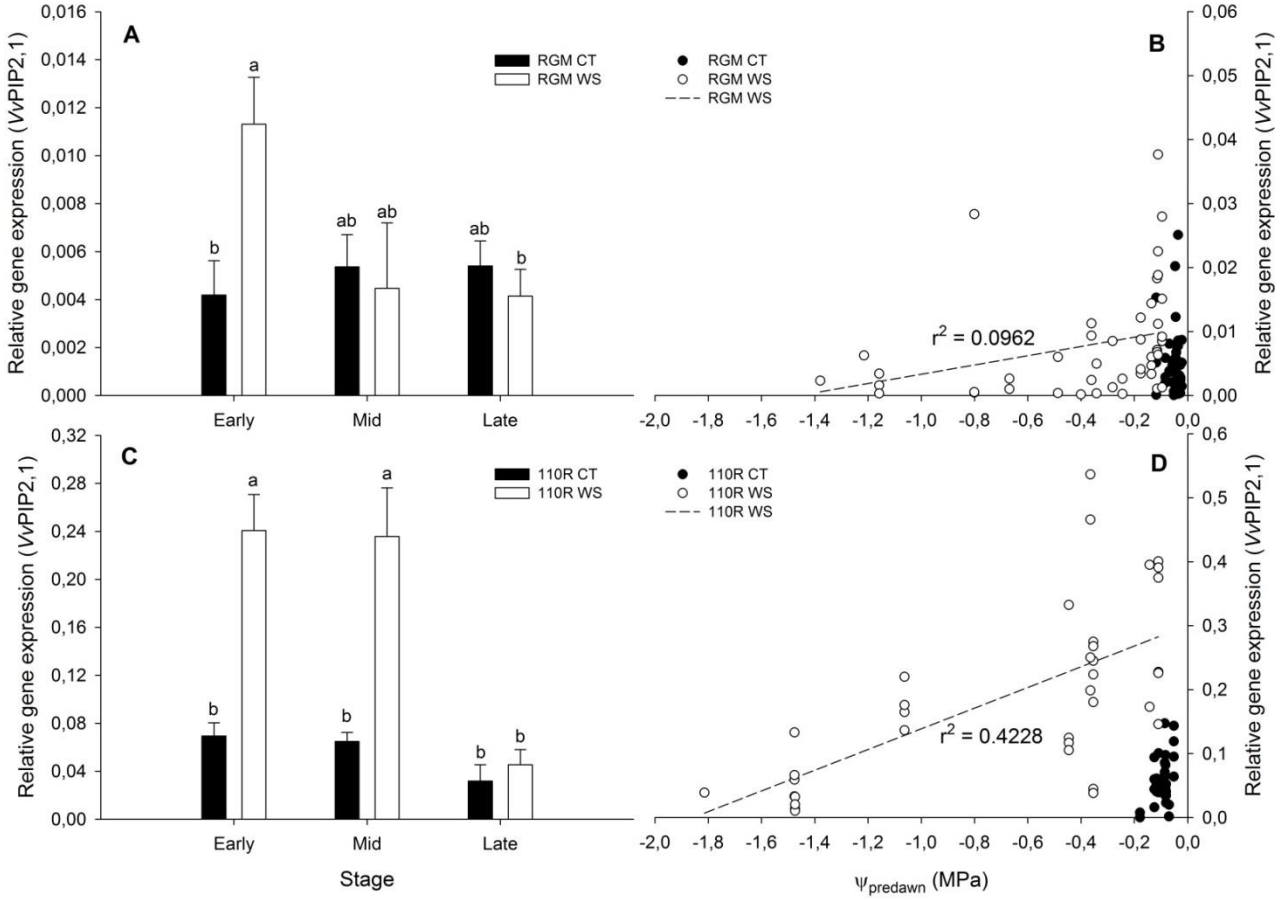

Figure 4.10 Average relative gene expression level of *Vv*PIP2,1 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP2,1 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP2,1 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP2,1 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

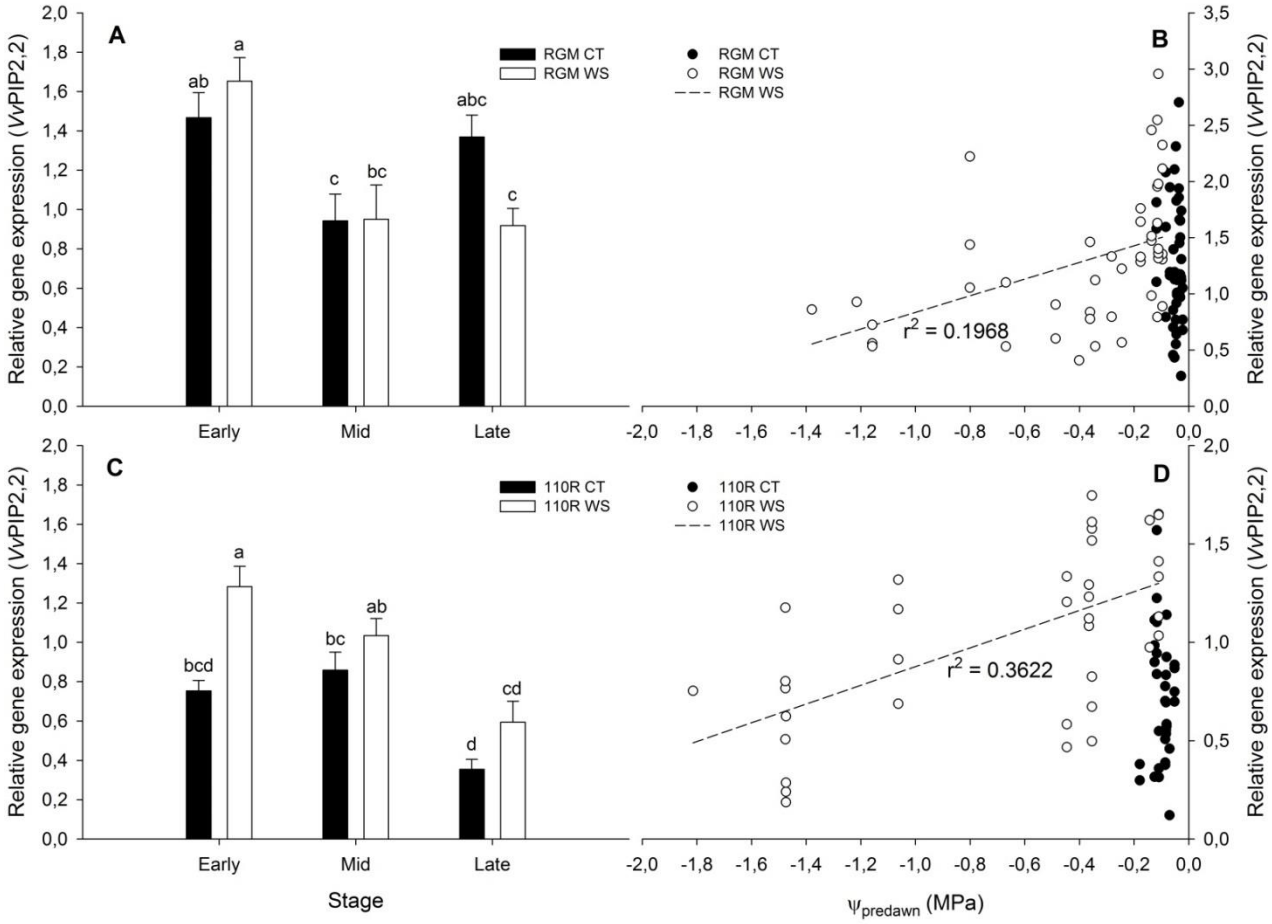

Figure 4.11 Average relative gene expression level of *Vv*PIP2,2 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP2,2 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP2,2 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP2,2 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

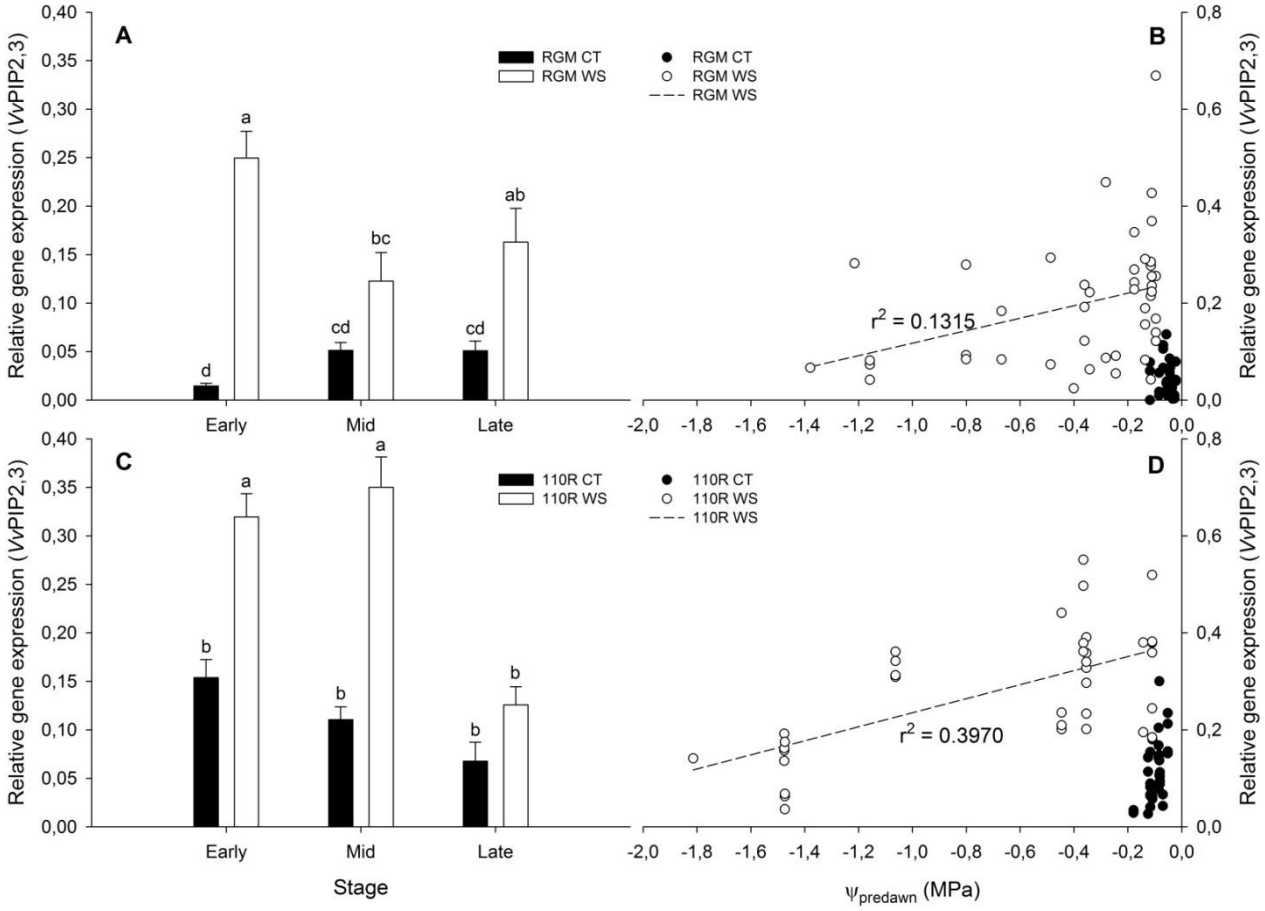

Figure 4.12 Average relative gene expression level of *Vv*PIP2,3 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP2,3 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP2,3 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP2,3 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

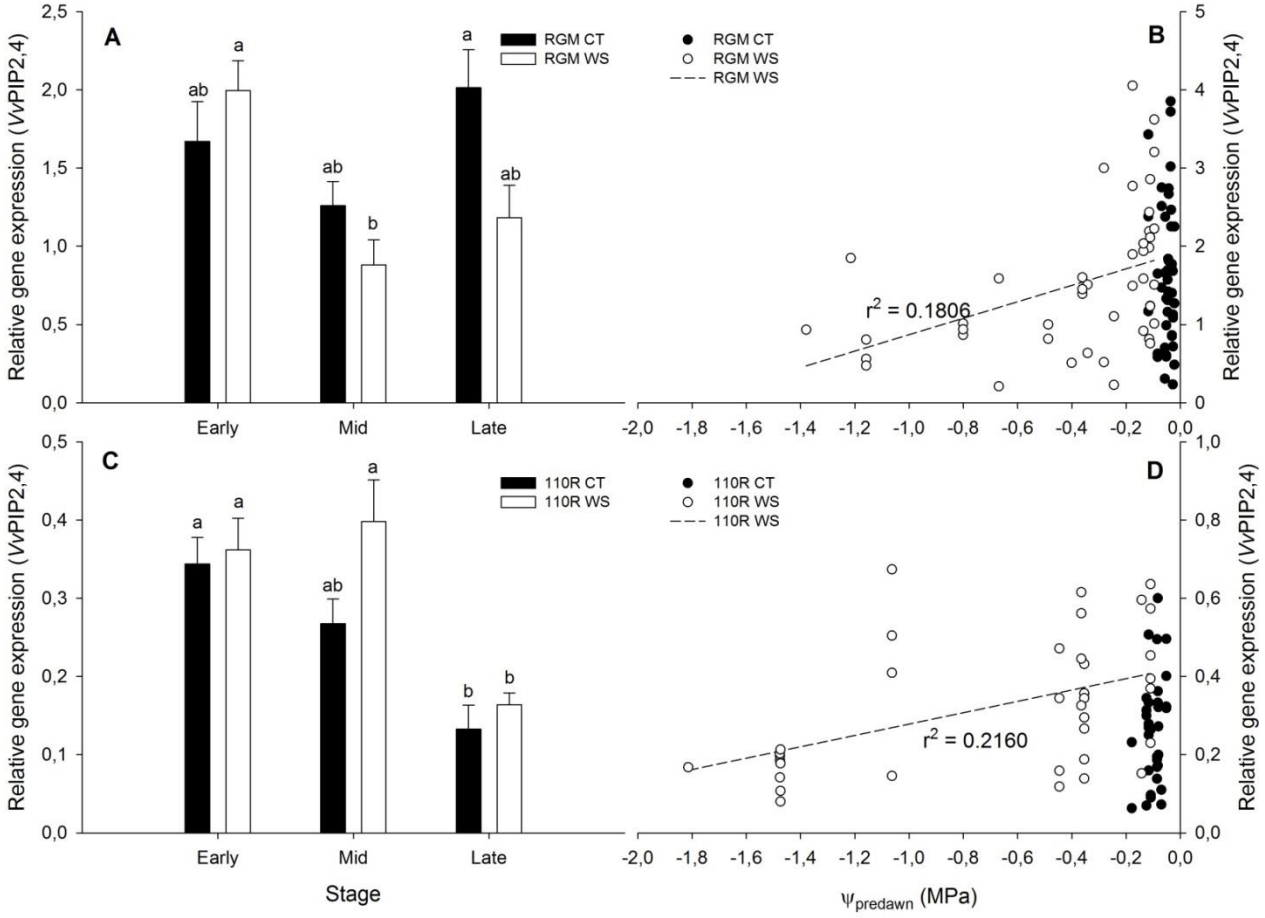

Figure 4.13 Average relative gene expression level of *Vv*PIP2,4 in response to water stress and plant developmental stage, and relationship between relative *Vv*PIP2,4 gene expression and pre-dawn leaf water potential for individual roots. A and C, average relative *Vv*PIP2,4 gene expression level of all individual roots across three developmental stages under both well-watered and water-stressed conditions for RGM and 110R, respectively. Values are mean ± SE (two-way ANOVA, Tukey's HSD test, $p < 0.05$, n-RGM-CT-Early = 17, n-RGM-CT-Mid = 15, n-RGM-CT-Late = 13, n-RGM-WS-Early = 22, n-RGM-WS-Mid = 10, n-RGM-WS-Late = 12, n-110R-CT-Early = 12, n-110R-CT-Mid = 15, n-110R-CT-Late = 7, n-110R-WS-Early = 15, n-110R-WS-Mid = 12, n-110R-WS-Late = 9). B and D, relationship between relative *Vv*PIP2,4 gene expression and values of $\Psi_{predawn}$ for all individual roots analyzed under both well-watered and water-stressed conditions for RGM and 110R, respectively.

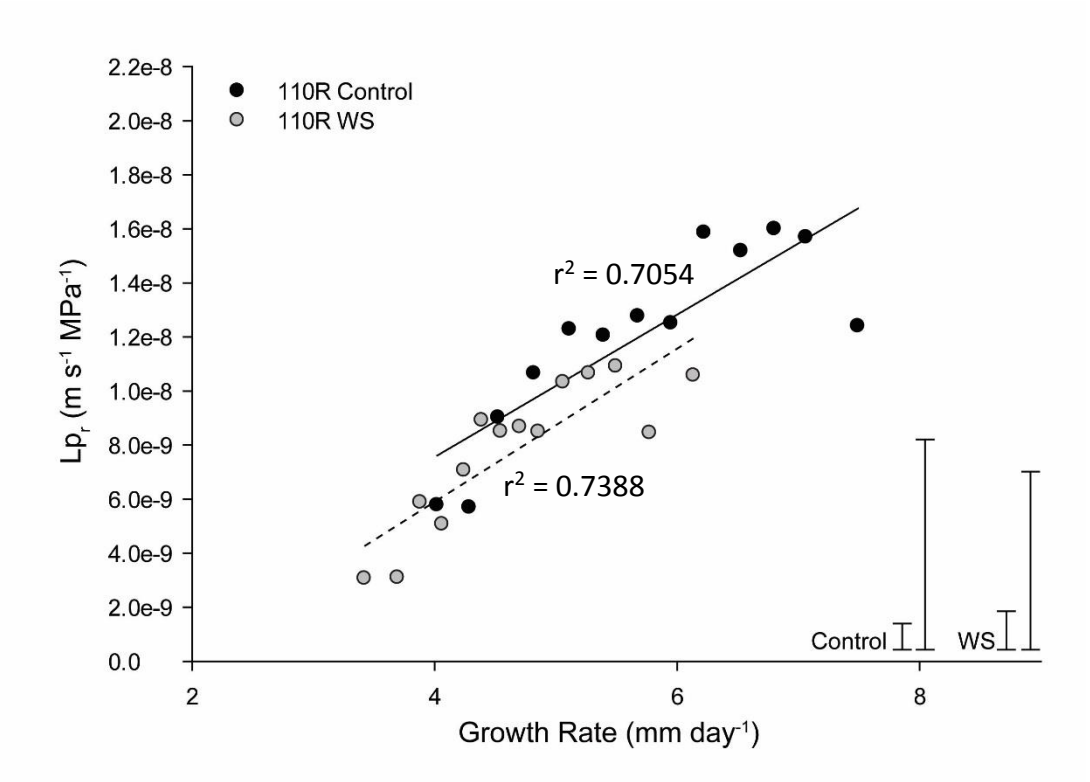

Figure 4.14 Relationship between Lp_r and root growth rate for 110R. Moving averages of 10 values from fastest to slowest growing roots of root growth rate and Lp_r for both well-watered (110R Control, black dots) and water-stressed (110R WS, grey dots) conditions were calculated and plotted in the graph. The ranges of SE for Lp_r Control and WS are presented on the bottom right of the graph. A linear regression line was fitted for both Control and WS respectively as significant correlation between Lp_r and root growth rate was found with GLM analysis ($p < 0.05$).

4.3.3 Relationship between root hydraulic conductivity and root growth rate

In order to investigate the relationship between root hydraulic conductivity and root growth rate for both RGM and 110R, moving averages of 10 values from fastest to slowest growing roots of root growth rate and Lp_r for both well-watered and water-stressed conditions were calculated and analyzed with GLM model (Figure 4.14). Significant correlation between the moving averages of Lp_r and root growth rate was found in 110R with $p = 0.047$, while no correlation was found in RGM.

4.3.4 Relationship between aquaporin gene expression and root growth rate

The relationship between the expression level of aquaporin genes and root elongation rate of all individual roots were investigated with GLM model and significant correlations were plotted. The expression of 3 PIP2 genes, *Vv*PIP2,1, *Vv*PIP2,2, and *Vv*PIP2,4, were demonstrated to be

correlated with root growth rate. However, only the changes of *Vv*PIP2,2 expression in response to root growth rate were consistent in all conditions. As presented in A and B in Figure 4.16, for both RGM and 110R, the expression level of *Vv*PIP2,2 was positively correlated with the speed of root growth under both well-watered and water-stressed conditions. The expression of *Vv*PIP2,1 was positively correlated with root growth in RGM under water stress (Figure 4.15). With regard to *Vv*PIP2,4, whether gene expression is correlated with root growth depends both on genotype and plant water status. A positive correlation was found in RGM under drought treatment (A in Figure 4.17), while in 110R, *Vv*PIP2,4 expression was only positively correlated with root growth in well-watered plants (B in Figure 4.17).

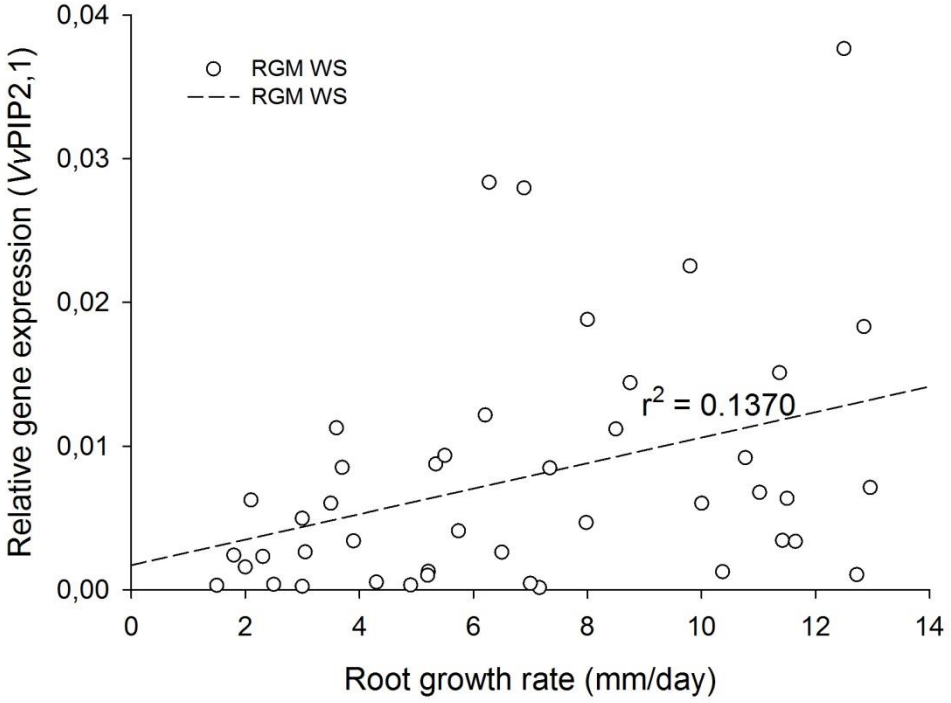

Figure 4.15 Relationship between the relative gene expression level of *Vv*PIP2,1 and root growth rate of individual roots for RGM under water stress. Linear regression line was fitted as significant correlation was found with GLM analysis between the relative gene expression level of *Vv*PIP2,1 and root growth rate in RGM under waters stress ($p < 0.05$).

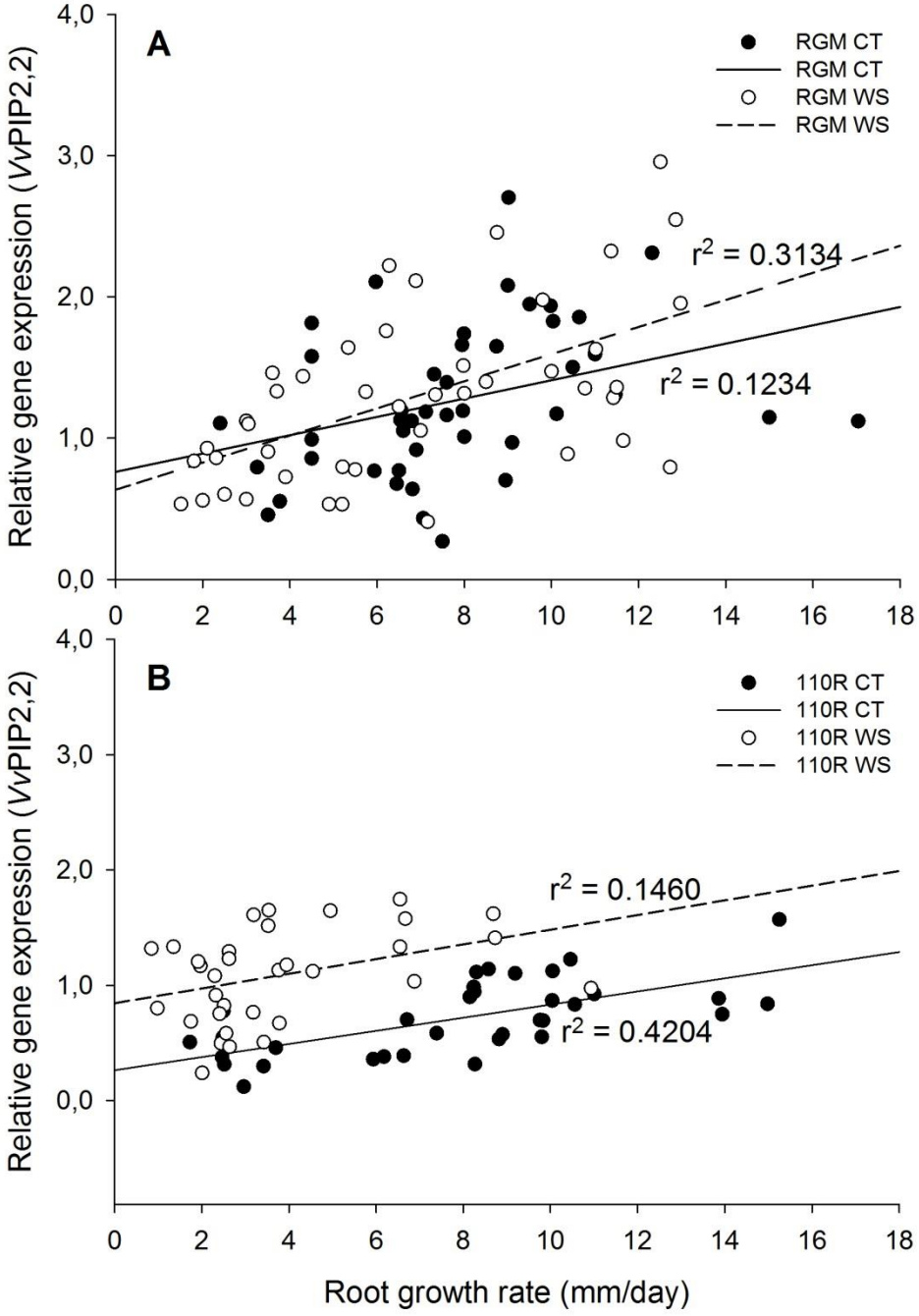

Figure 4.16 Relationship between the relative gene expression level of *Vv*PIP2,2 and root growth rate of individual roots. A and B represent data for both well-watered and water-stressed roots from RGM and 110R, respectively. Linear regression lines were fitted as significant correlations were found with GLM analysis between the relative gene expression level of *Vv*PIP2,2 and root growth rate for all conditions ($p < 0.05$).

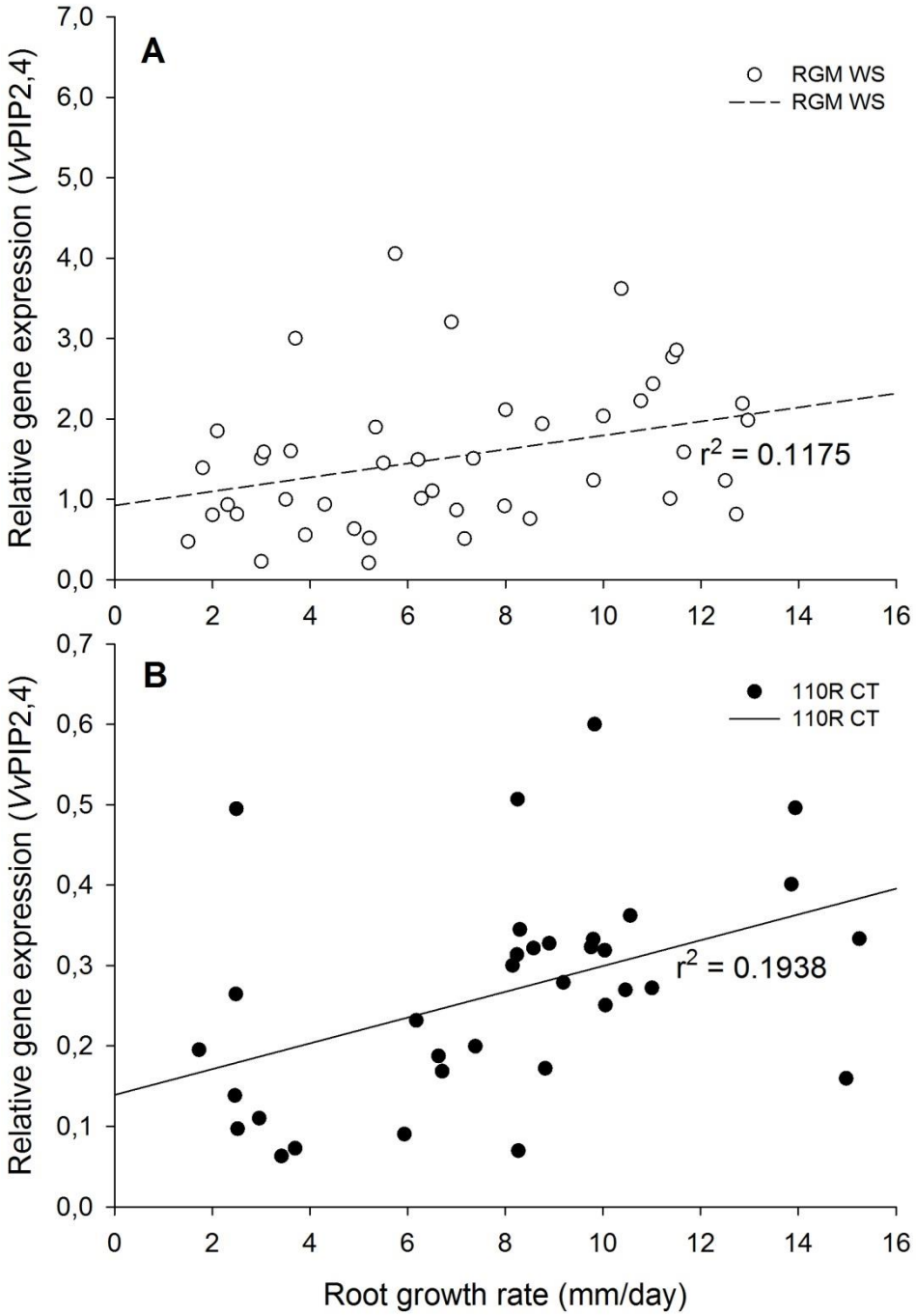

Figure 4.17 Relationship between the relative gene expression level of *Vv*PIP2,4 and root growth rate of individual roots. A and B represent data for water-stressed roots from RGM and well-watered roots from 110R, respectively. Linear regression was fitted as significant correlation was found with GLM analysis between the relative gene expression level of *Vv*PIP2,4 and root growth rate for each condition ($p < 0.05$).

4.4 Discussions

4.4.1 Root hydraulic conductivity and the impact of water stress

The osmotic Lp_r of individual grapevine fine roots exhibited high plasticity even within one genotype and one treatment during the period of one experiment. Table 4.6 shows a summary of the minimum and maximum Lp_r values measured across three experiments carried out. For well-watered roots, the interspecies Lp_r variances can be 15- to 20-fold depending on the variety and experiment, while for water-stressed roots, the differences can be 25- to 110-fold depending on the variety and experiment. As the data of root hydraulic conductivity were collected over a long growing period and across a large number of roots, this can be one reasonable explanation for the observed large variability. Gambetta *et al.* (2012) have pointed out a high variability in Lp_r values across individual grapevine rootstock roots as well. Moreover, plants are known for their capacity of continuous physiological and structural adjustment during their lifetime to optimize viability facing various environmental constraints (Plavcová and Hacke 2012). Thus, plant functional and structural traits are characterized by phenotypic plasticity (von Arx *et al.* 2012, Plavcová and Hacke 2012), and those related to water balance may play a critical role in determining plant performance under water deficit conditions especially for perennial species (von Arx *et al.* 2012). North and Nobel (1998) investigated water uptake and structural and hydraulic plasticity along roots of a desert succulent during prolonged drought and rewatering and pointed out that anatomical or developmental plasticity within individual roots affected water uptake by the root system after drought. By evaluating a panel of 41 soybean accessions, Prince *et al.* (2017) reported that owing to root xylem developmental plasticity, increases in metaxylem number as an adaptation to drought in the high-yielding lines improved root hydraulic conductivity. In addition to inherent structural and physiological plasticity, Carminati and Vetterlein (2012) introduced another concept of plasticity which implies soil and plant water relations at the plant scale, the rhizosphere plasticity, as root functions rely on soil properties as well. According to Carminati and Vetterlein (2012), rhizosphere plasticity is the result of several processes including root and soil shrinking/swelling during drying/wetting cycles, soil compaction by root growth, mucilage exuded by root caps, interaction of mucilage with soil particles, mucilage shrinking/swelling and mucilage biodegradation. The hydraulic properties of the rhizosphere can obviously influence root hydraulic conductivity and root water uptake. In our experiments, rhizosphere properties such as the level of soil compaction and mucilage related attributes can possibly be modified during the period of each experiment due to irrigation/drying-down process and root growth and development, which may contribute to the observed plasticity in Lp_r of individual fine roots.

Table 4.6 Minimum and maximum Lp_r values obtained in all three experiments

	CT (m/s/MPa)		WS (m/s/MPa)	
	Min	**Max**	**Min**	**Max**
RGM May-June 2016	3.28E-9	7.08E-8	1.18E-9	3.04E-8
110R May-June 2016	2.88E-9	4.23E-8	3.32E-10	2.94E-8
110R August-September 2016	1.82E-9	2.71E-8	7.97E-11	8.89E-9

As presented in panel A in Figure 4.2, 4.3, and 4.4, Lp_r declined significantly in mid and late stages in contrast to early stage within the growing period of each experiment in well-watered roots. As a matter of fact, this drop in Lp_r with time accounted for a large part of the plasticity of individual root hydraulic conductivity mentioned above. Given the environmental conditions to which the plants were exposed in our experiment were relatively constant in the greenhouse, it is suggested that decreases in fine root Lp_r with time going on were associated with root developmental process which is age-related. In terms of appearance, fine roots are white in the beginning of their life span, then they may become brown with aging and remain in this state (Richards and Considine 1981, Hendrick and Pregitzer 1992, McKenzie and Peterson 1995, Wells and Eissenstat 2003). Many changes in the physiological functions of fine roots can be related to aging such as nutrient uptake and root respiration, so does root anatomical structure (Wells and Eissenstat 2003). Alterations in fine root hydraulic conductivity with aging have been reported as well, with implications for both water uptake and nutrient uptake (Kramer 1983, Wells and Eissenstat 2003). In grapevine roots, whose median lifespan is approximately 50-70 days, a reduction of 70% of maximum nitrate uptake capacity within 10 days was observed in rootstock variety 3309C (A. Volder unpublished data, Wells and Eissenstat 2003). Then there was no obvious decrease in nitrate absorption until day 23 (A. Volder unpublished data, Wells and Eissenstat 2003). This result is actually consistent with what we observed in our experiments regarding root water uptake. Fine root hydraulic conductivity decreased drastically from early stage to mid stage while remained constant between mid and late stages for both rootstock varieties. Similarly, Nobel *et al.* (1990) have reported decreased root hydraulic conductivity with aging in a desert succulent Engelm (*Agave deserti*) and observed an approximately linear decline of individual roots hydraulic conductivity with aging from 2 weeks to 3 months. However, the pattern of changes in root hydraulic conductivity with aging may differ between plant species. For example, root hydraulic conductivity increased with aging until 11-17 weeks and then decreased with aging afterwards in *Ferocactus acanthodes* and *Opuntia ficus-indica* species (Oosterhuis 1983, Drew 1987, Wells and Eissenstat 2003).

The osmotic Lp_r values of well-watered individual fine roots of RGM and 110R are in a similar range to that measured by Gambetta *et al.* (2012) in grapevine rootstocks 420A and 110R with the same method, even though the Lp_r values in our experiments are a little bit higher sometimes with a magnitude of 10^{-8} while in the experiment of Gambetta *et al.* (2012) the osmotic Lp_r values are in the magnitude of 10^{-9}. However, this should not be surprising as mentioned earlier that plants exhibit high plasticity in their physiological functions. Interestingly, the osmotic Lp_r values of individual fine roots are also in a similar range with the Lp_r values of the whole root system normalized by root surface area of four grapevine varieties determined by the high pressure flow meter (HPFM) from Vandeleur (2007). In a study conducted by Nobel and Huang (1992) in two desert succulents, root hydraulic conductivity was reduced in response to drought stress and this decrease was similar between the whole root systems of intact plants and the excised roots. The value of root hydraulic conductivity also depends on the nature of the driving force applied in the measurement. In general, measured root hydraulic conductivity is typically higher under a hydrostatic driving force compared with an osmotic one (Knipfer and Fricke 2011, Gambetta *et al.* 2012, 2013), because under a hydrostatic pressure gradient, water is driven through both apoplastic and cell-to-cell pathways, while under an osmotic pressure some portion of the pathway is cell-to-cell (Gambetta *et al.* 2013). Furthermore, the value of root hydraulic conductivity also varies according to the parameter used to normalize root hydraulic conductance. Normally, values of root hydraulic conductance could be scaled by dividing by some measures of root size (e.g., root surface area, total root length, or root mass) or by leaf surface area to calculate root hydraulic conductivity (Tyree *et al.* 1998). For example, in grapevine, no difference was observed between four different varieties studied when root system hydraulic conductance was normalized by root dry weight; however, differences appeared with normalization by leaf area and root surface area (Vandeleur 2007). This reveals an involvement of root surface area to dry weight ratio, which ultimately concerns the influence of root morphology on root hydraulic conductance and water uptake (Vandeleur 2007). In our experiment, only one parameter, root surface area of the individual root, is used in the normalization of root hydraulic conductance.

Fine root Lp_r decreased significantly when plants experienced drought stress in the early growing stage in each experiment performed. Although for 110R from the experiment conducted in May 2016, the Lp_r decrease of about 40% in the early stage was not statistically significant possibly due to high variability of Lp_r measured across individual roots. However, in mid and late growing stages, even though we can see a trend of slight decrease in Lp_r of water-stressed roots, this decrease was nearly negligible and statistically there was no difference in Lp_r between well-watered and water-stressed roots. As mentioned previously, we attribute this phenomenon to plant aging. Therefore, fine root Lp_r in grapevine declines even under well-watered conditions with progressed developmental stage. Moreover, Melchior and Steudle (1993) studied the changes in radial hydraulic conductivity during root development in onion (*Allium cepa* L.) and discovered that Lp was smaller and more variable in more basal zones of the root due to more developed exodermal Casparian bands and/or suberin lamellae in the endodermis or exodermis.

This supports our observation which root hydraulic conductivity declines with developmental stages even under well-watered conditions.

Nevertheless, it is common to observe a decreased root hydraulic conductivity when plants are exposed to drought constraints as demonstrated by numerous studies across various species. Among perennial plants, Rieger (1995) reported reductions in root hydraulic conductivity to varying degrees in peach (*Prunus persica* L. Batsch), olive (*Olea europaea* L.), citrumelo (*Poncirus trifoliata* Raf x *Citrus paradisi* Macf.) and pistachio (*Pistachia integerrima* L.) and Trifilo *et al.* (2004) reported decreased root hydraulic conductivity in ailanthus (*Ailanthus altissima*). Down-regulations of root hydraulic conductivity under water stress have also been observed in annual herbaceous plant species such as common bean (*Phaseolus vulgaris*, Aroca *et al.* 2006), lettuce (*Lactuca sativa*, Aroca *et al.* 2008), and rice (*Oryza sativa* L., Gao *et al.* 2010). A large amount of studies has been carried out in desert plant species such as *Agave deserti* (North and Nobel 1998, 2000, 2004), *Opuntia ficus-indica* L. (North and Nobel 1992, 1996), *Ferocactus acanthodes* (North and Nobel 1992), *Opuntia acanthocarpa* (Martre *et al.* 2001). In grapevine, Vandeleur (2007) observed significant decreases in whole root system hydraulic conductivity under drought in two scion cultivars Chardonnay and Grenache and one rootstock variety 101-14. Barrios-Masias *et al.* (2015) evaluated fine root hydraulic conductivity under different moisture conditions and across different rootstock varieties and found that Lp decreased for both 101-14 and 110R under dry conditions with a hydrostatic driving force. However, with an osmotic driving force, reductions in Lp under dry conditions were only observed in 101-14. Although decreases in root water uptake and root hydraulic conductivity are generally observed in roots exposed to drought, increases in Lp have been observed under certain specific circumstances. For example, Siemens and Zwiazek (2004) reported an up-regulation in root Lp in solution culture-grown aspen (*Populus tremuloides*) seedlings subjected to mild water stress by being exposed to a sealed high humidity chamber for 17 hours. However, conversely, root hydraulic conductivity was reduced in roots under severe water stress. The initial decrease of hydraulic conductivity upon roots exposure to drought constraints is suggested to be a protective mechanism to prevent water from leaking back to soil with an increasingly negative water potential and lower than that of the roots (Vandeleur 2007, Aroca *et al.* 2011).

RGM and 110R were selected in our experiment due to their distinct drought resistance capacity with 110R being more resistant to drought stress. It would be interesting to find out if higher drought resistance in 110R is related to higher root water uptake capacity and higher root hydraulic conductivity or not. However, as shown in Figure 4.2, 4.3, and 4.4, as well as the summary of minimum and maximum Lp_r values in Table 4.6, apart from the large variability, no obvious difference was observed in root hydraulic conductivity between RGM and 110R. Rieger (1995) conducted measurements of root hydraulic conductivity in several tree species differed in inherent tolerance to drought but found no differences in Lp under well-watered conditions. In grapevine, Vandeleur (2007) has reported the lowest Lp value observed in the drought-tolerant variety Grenache, while the highest Lp value was measured in a less drought tolerant variety

Chardonnay. However, no evident trend between root hydraulic conductivity and drought tolerance could be defined within the four grapevine varieties examined. Peccoux (2011) has reported significantly higher single root hydraulic conductivity in RGM compared with 110R regardless of water supply. Moreover, drought treatment did not modify single root hydraulic conductivity in both RGM and 110R (Peccoux, 2011). Nonetheless, the inconsistent results in whether or not there are differences in individual root hydraulic conductivity in RGM and 110R between our experiment and the one from Peccoux (2011) could possibly result from the measuring methodology and the different parameters used for the normalization of the Lp data. In sugarcane (*Saccharum* spp. hybrid), Saliendra and Meinzer (1989) have reported the highest apparent root hydraulic conductance (calculated from transpiration rate and hydrostatic pressure gradients) being determined in the most drought-resistant cultivar. Nevertheless, it seems that no consistent relationship between root hydraulic conductivity and drought resistance has yet been found. Thus, root hydraulic conductivity may not be a good parameter for the evaluation of plant resistance to drought.

4.4.2 The role of aquaporins in the regulation of root hydraulic conductivity

The rate of root taking up water is characterized by root hydraulic conductivity (Aroca *et al.* 2011), and many studies have revealed that root hydraulic conductivity is directly associated with root water uptake rate (Nobel and Alm 1993, Gallardo *et al.* 1996, Nardini and Pitt 1999). Aquaporins have been discovered in plants (Maurel *et al.* 1993) as membrane intrinsic proteins which facilitate the transport of water across plasma membranes, and the role of aquaporins in plant water uptake has been investigated vastly. From this aspect, the contribution of aquaporins to root hydraulic conductivity has received appreciable attention, and different approaches have been applied in order to determine the portion of root hydraulic conductivity which aquaporins are accounted for (Aroca *et al.* 2011).

Studies using molecular tools have demonstrated the involvement of aquaporins in root water uptake and their importance to root hydraulic conductivity. Antisense suppression of PIP1 aquaporin in tobacco transgenic plant resulted in reduced root hydraulic conductivity and lower resistance to water stress (Siefritz *et al.* 2002). Two Arabidopsis knockout mutants of aquaporin PIP2,2 showed decreased osmotic root hydraulic conductivity and root cortex cell hydraulic conductivity compared with wild type (Javot *et al.* 2003). Similarly, knocking out aquaporin PIP1,2 in Arabidopsis declined hydrostatic root hydraulic conductivity but did not modify osmotic root hydraulic conductivity (Postaire *et al.* 2010). Lovisolo *et al.* (2007) have reported a higher root hydraulic conductance associated with a higher aquaporin gene expression level in a perennial woody plant olive (*Olea europaea* L.).

To assess the functional contribution of aquaporins to root hydraulic conductivity, several non-specific chemical inhibitors have been tested and applied. In the beginning, the inhibitory treatments in hydraulic conductivity were mostly realized with mercurial reagents (Maggio and Joly 1995, Carvajal *et al.* 1996, Zhang and Tyerman 1999, Postaire *et al.* 2010, Sutka *et al.* 2011).

The aquaporin inhibitors are not restricted to Hg. Other heavy metals such as silver and gold (Niemietz and Tyerman 2002), cytosolic pH regulator with weak acids such as sodium azide (NaN_3), propionic acid, potassium cyanide and sodium acetate (Tournaire-Roux *et al.* 2003, Postaire *et al.* 2010, Sutka *et al.* 2011) and hydrogen peroxide (Aroca *et al.* 2005, Boursiac *et al.* 2008, Gambetta *et al.* 2012) have also been used in many studies. These studies have demonstrated that aquaporins can attribute up to 30% to 80% of root hydraulic conductivity (North *et al.* 2004, Sutka *et al.* 2011, Pou *et al.* 2013). In our study, 1 mM NaN_3 was used as an inhibitor in order to obtain a better understanding in to what extent aquaporins contribute to root hydraulic conductivity. We selected NaN_3 as an inhibitor due to its demonstrated efficiency (e.g., Tournaire-Roux *et al.* 2003, Sutka *et al.* 2011) and its lower toxicity compared with another commonly used inhibitor Hg. Ideally, we would have liked to repeat the experiments with different inhibitors; however, practically, as the measurements are extremely laborious, doubling the number of experiments is not feasible. 1 mM NaN_3 as a weak acid regulates aquaporin gating by influencing cytosolic pH and further blocking respiration via the cytochrome pathway (Tournaire-Roux *et al.* 2003). Previous studies have demonstrated NaN_3 to be an effective inhibitor to aquaporin activity with an inhibition level of 77% ± 2% (Sutka *et al.* 2011) or even up to 87% ± 1% (Tournaire-Roux *et al.* 2003). Similar to the values of individual root hydraulic conductivity, percentage of inhibition in Lp_r also showed high variability within one genotype and one treatment, e.g., for RGM, relative inhibition levels were 25%-88% and 4%-93% in well-watered and water-stressed plants, respectively; for 110R, relative inhibition levels were 40%-95% and 6%-93% in well-watered and water-stressed plants, respectively (two 110R experiments combined). Possibly due to the huge variations in relative inhibition, in most cases, percentage of inhibition on average did not differ significantly between neither treatments nor developmental stages for both varieties (panel B in Figure 4.2, 4.3, and 4.4). Root hydraulic conductivity and residual Lp_r after inhibition were plotted in panel A in Figure 4.2, 4.3, and 4.4 and the difference between them is the inhibitable Lp_r component. We consider this to represent the metabolic contribution to Lp_r which would include aquaporins, but may also include other mechanisms like changes in cell water relations such as turgor which could potentially impact the resistance of the pathway. Generally, for both varieties, in mid and late developmental stages, no significant differences were observed in root hydraulic conductivity and relative inhibition level between well-watered and water-stressed roots. Therefore, we can assume that during mid and late stages, the aquaporin-dependent and aquaporin-independent pathways are similar between well-watered and water-stressed plants. However, in the early stage, the absolute inhibitable Lp_r components seem much higher in well-watered than in water-stressed roots (panel A early stage in Figure 4.2 and 4.4). Thus, aquaporin-dependent pathways account for a greater part in well-watered plants in the beginning of the development process. In a similar study conducted by Grondin *et al.* (2016) in six diverse rice (*Oryza sativa* L.) varieties, NaN_3 was also chosen as an aquaporin inhibitor and changes in contribution of aquaporins to root hydraulic conductivity under drought stress were variety-dependent. Significantly reduced, significantly increased, and not changed aquaporin contribution to Lp_r in response to drought stress were observed in two out of six varieties studied, respectively. Pou *et al.* (2013) investigated the role of aquaporins in leaf

hydraulic conductance in grapevine and suggested that under water stress apoplasmic pathways became more important.

By using a fluorescent tracer dye 3-hydroxy-5,8,10-pyrenetrisulfonate (PTS_3), Siemens and Zwiazek (2003) observed a greater proportion of apoplastic root water flow in severely stressed plants, which is another approach to estimate the proportion of apoplastic/symplastic water flux and to determine the potential role of aquaporins in root water transport (Hanson *et al.* 1985, Moon *et al.* 1986, Skinner and Radin 1994, Pou *et al.* 2013). PTS_3 is a non-ionic fluorescent dye and does not enter across cell membranes, so it is used as an apoplastic tracer (Siemens and Zwiazek 2003).

In the short term, changes in root hydraulic conductivity have been largely proved to be mediated through the regulation of aquaporin expression and activity (Maurel *et al.* 2010). Root hydraulic conductivity has been observed to fluctuate diurnally and this diurnal regulation has been associated with aquaporin gene expression (Henzler *et al.* 1999, Clarkson *et al.* 2000, McElrone *et al.* 2007, Vandeleur *et al.* 2009, Sakurai-Ishikawa *et al.* 2011). The important role of aquaporins in root hydraulic conductivity as well as the recovery of root hydraulic conductivity from external constraints such as water deficit and chilling has been investigated in detail (e.g., Martre *et al.* 2002, North *et al.* 2004, Aroca *et al.* 2005). In the long term, root growth and development can be strongly affected by external stimuli ending up with modified anatomical structure and even modified root system architecture; thus, root hydraulics may be regulated on another level (Maurel *et al.* 2010). For instance, under water-stressed conditions, higher degree of suberization was observed in both exodermis and endodermis in the root tip, which could reduce root permeability to water due to the hydrophobic property of suberin lamellae (Zimmermann *et al.* 2000, Vandeleur *et al.* 2009, Barrios-Masias *et al.* 2015). Additionally, coupled with structural changes of roots in response to environmental stresses, abundances of aquaporin gene transcripts may be influenced as well (Maurel *et al.* 2010).

In addition to its function in controlling root growth and development under environmental stresses, the role of ABA has also been recognized in the regulation of root hydraulic conductivity and aquaporin transcript abundance and activity in plants facing drought stress, even though no consistent conclusions have been reached. As mentioned earlier, water stress tends to decrease root hydraulic conductivity, while in general it is believed that ABA has an opposite effect and thus can improve root water uptake under environmental constraints (Parent *et al.* 2009, Sánchez-Romera *et al.* 2014). In order to understand the potential role of ABA in the regulation of root hydraulic conductivity, studies have been conducted with exogenous ABA application or with manipulating endogenous ABA amount in transgenic plants (Sánchez-Romera *et al.* 2014). However, the relationship between ABA and root hydraulic conductivity is controversial as positive (Glinka and Reinhold 1971, Hose *et al.* 2000, Schraut *et al.* 2005, Mahdieh and Mostajeranb 2009, Parent *et al.* 2009, Kudoyarova *et al.* 2011), negative (Markhart *et al.* 1979, Wan and Zwiazek 2001, Vandeleur 2007), as well as no effect (Cram and Pitman 1972, Erlandsson *et al.* 1978, Aroca *et al.* 2003) have all been reported. As a matter of fact, impacts

exerted by ABA on root hydraulics are dependent on the duration, ABA concentration, plant species, and growth environment, etc. (Markhart *et al.* 1979, Maurel *et al.* 2010, Dodd 2013). Mechanisms involved in the ABA-dependent regulation of aquaporin activity can be either at the transcriptional level by changing aquaporin gene expression or at the post-transcriptional level e.g. by possible gating of existing aquaporins (Kaldenhoff *et al.* 2008, Sharipova *et al.* 2016). ABA may also induce transcription factors to alter the expression of aquaporin genes (Kaldenhoff *et al.*1996, Shinozaki *et al.* 1998). Normally, an up-regulation of PIP gene expression can be observed with exogenous application of ABA (Jang *et al.* 2004, Lian *et al.* 2006). Increased endogenous ABA level resulted from salt stress was associated with induced expression of several PIP isoforms in maize (*Zea mays* L. cv. Helix) (Zhu *et al.* 2005). Exogenous application of ABA at 1 µM transiently induced the expression of similar PIP isoforms and confirmed the up-regulation of PIP genes mediated by ABA (Zhu *et al.* 2005). However, high level of exogenous ABA (100 µM) supply completely suppressed the expression of PIP and TIP genes examined (Zhu *et al.* 2005). Compared with wild type, sense maize plant producing higher endogenous ABA had higher PIP gene expression level and PIP protein amount in roots as well as accompanied higher root hydraulic conductance, while antisense plants showed completely opposite results (Parent *et al.* 2009). In barley, an ABA-deficient mutant Az34 exhibited lower level of endogenous ABA, lower root hydraulic conductivity, but similar expression level of PIP genes compared with wild type (Sharipova *et al.* 2016). External apply of ABA to Az34 up-regulated ABA concentration and aquaporin abundance in root cells, and in the meantime increased both root hydraulic conductivity and cortical cell hydraulic conductivity (Sharipova *et al.* 2016). In our experiments, it would be interesting to examine the ABA concentration in roots, which may give more insights regarding changes in root hydraulics and aquaporin activities in response to water stress as well as along plant developmental stages.

4.4.3 *Vv*PIP aquaporin gene expression

In general, plasma membrane intrinsic proteins (PIPs) account for the most abundant aquaporins in root plasma membrane and play a central role in mediating transcellular root water uptake (Tournaire-Roux *et al.* 2003, Sutka *et al.* 2011). As previously indicated, changes in root hydraulic conductivity can be partially regulated by the activity of aquaporins, and in particular PIPs (Javot *et al.* 2003, Postaire *et al.* 2010). Therefore, the expression of 7 PIP aquaporin genes in fine root tips, including 3 genes (*Vv*PIP1,1, *Vv*PIP1,2,4, and *Vv*PIP1,3,5) from PIP1 subfamily and 4 genes (*Vv*PIP2,1, *Vv*PIP2,2, *Vv*PIP2,3 and *Vv*PIP2,4) from PIP2 subfamily, were analyzed in our experiment. These genes selected have been previously defined by Gambetta *et al.* (2012) based on all available *Vv*PIP gene sequences identified by Shelden *et al.* (2009) and examined in different grapevine rootstocks. Grapevine PIP genes are highly conserved at the DNA level, particularly, for example, PIP1,2 and PIP1,4 are 98% identical at the DNA level, and PIP1,3 and PIP1,5 are 96% identical at the DNA level (Shelden *et al.* 2009). Therefore, for these isogenes, the expression data are presented as *Vv*PIP1,2,4 and *Vv*PIP1,3,5 in our experiment.

In our study, *Vv*PIP1,1 is the most expressed PIP1 gene and also the most expressed PIP gene among 7 genes analyzed. This is consistent with the expression data from Gambetta *et al.* (2012) and Rossdeutsch (2015). However, for the other PIP genes, the expression levels are less predictable. For more than half of the genes, the expression levels in RGM and 110R were comparable and fell in the same range. *Vv*PIP1,3,5 showed a much higher expression level in RGM than in 110R. For RGM, *Vv*PIP1,3,5 had a similar expression level with *Vv*PIP1,2,4, which is normally the second most expressed PIP1 gene in grapevine roots (Gambetta *et al.* 2012, Rossdeutsch 2015), under well-watered conditions. For 110R, *Vv*PIP1,3,5 showed a very low level of expression and was the least expressed PIP1 gene. *Vv*PIP2,4 also exhibited a much higher expression level in RGM than in 110R, making it the most expressed PIP2 genes in RGM together with *Vv*PIP2,2, which is often highly expressed in roots of grapevine rootstock varieties (Gambetta *et al.* 2012, Rossdeutsch 2015), and in our experiment was also the most expressed PIP2 gene in 110R. On the contrary, *Vv*PIP2,1 was much more expressed in 110R than RGM; nonetheless, it was the least expressed PIP2 gene for both varieties, which is inconsistent with the expression data with Gambetta *et al.* (2012) and Rossdeutsch (2015), who have reported *Vv*PIP2,3 and *Vv*PIP2,4 as the least expressed PIP2 gene, respectively. However, the expression profiles of *Vv*PIP1,1, *Vv*PIP2,1, *Vv*PIP2,2, *Vv*PIP2,3, and *Vv*PIP2,4 in grapevine rootstock varieties observed in Gambetta *et al.* (2012), Rossdeutsch (2015), as well as in our experiment disagree to a large extent with those reported in another grapevine variety 'Brachetto' (*Vitis vinifera*) (Perrone *et al.* 2012).

Apparently, no consistent trend was found in the changes of *Vv*PIP gene expression levels in response to water stress and developmental stages. Looking at many studies, this is often the case as down-regulated, up-regulated, and unchanged PIP gene expression have all been reported. In our experiment, changes in aquaporin expression under drought stress depend largely on the specific gene, the genotype, as well as the developmental stage. And in all cases, PIP genes evaluated in our experiment were either up-regulated or unaltered under drought stress depending on the gene, the genotype, and the developmental stage. Under well-watered conditions, for RGM, the expression level of most PIP genes did not change across different developmental stages, except for *Vv*PIP1,1, whose expression level was up-regulated in late stage; for 110R, two PIP1 genes and two PIP2 genes did not respond to developmental stages, while *Vv*PIP1,2,4, *Vv*PIP2,2 and *Vv*PIP2,4 were down-regulated across developmental stages or in late stage. Water stress did not modify the expression levels of *Vv*PIP2,2 and *Vv*PIP2,4 in RGM and *Vv*PIP1,1, *Vv*PIP1,2,4, and *Vv*PIP2,4 in 110R. Interestingly, when water stress causes an up-regulation in PIP gene expression, this often happens in early and/or mid stages of the growing season, apart from *Vv*PIP1,2,4, *Vv*PIP2,3 in RGM, which were up-regulated during both early and late developmental stages. For RGM, *Vv*PIP1,1, *Vv*PIP1,3,5, and *Vv*PIP2,1 were all up-regulated in early stage, and for 110R, *Vv*PIP1,3,5 and *Vv*PIP2,2 were up-regulated in mid and early stages, respectively, and *Vv*PIP2,1 and *Vv*PIP2,3 were up-regulated in both early and mid stages.

In common bean (*Phaseolus vulgaris* L. cv. Borlotto), after 4 days without watering, the expression levels of *Pv*PIP1;3 and *Pv*PIP2;1 genes were elevated, while the expression of *Pv*PIP1;2 and *Pv*PIP1;1 was drastically decreased and remained unchanged, respectively (Aroca *et al.* 2007). In maize (*Zea mays* L. cv. Potro), after 4 days withholding water, 7 *Zm*PIP genes examined were also differentially regulated: the expression of *Zm*PIP1;1 gene was up-regulated, the expression of ZmPIP2;5 and 2;6 genes was down-regulated, and the expression of *Zm*PIP1;2, 1;5, 2;1, and 2;2 genes maintained constant (Ruiz-Lozano *et al.* 2009). In tobacco (*Nicotiana tabacum* cv. Samsun), drought stress significantly decreased the transcript abundance of *Nt*PIP1;1 and *Nt*PIP2;1 genes while increased the transcript abundance of *Nt*AQP1 gene (Mahdieh *et al.* 2008). In two grapevine scion varieties, the expression of *Vv*PIP2;2 gene was not modified under water stress in both varieties, while the expression of *Vv*PIP1;1 gene was up-regulated in Chardonnay but remained unchanged in Grenache (Vandeleur *et al.* 2009). Galmés *et al.* (2007) investigated the changes in *Vv*PIP gene expression in response to water stress in 110R and observed similar trend as in our experiment: *Vv*PIP gene expression varies depending on the gene as well as the stress level. Under high level of stress, the expression levels of *Vv*PIP1;3 and *Vv*PIP2;2 genes were up-regulated while the expression levels of *Vv*PIP1;1, 1;2, and 2;1 did not change. Based on the expression data, it is difficult to clarify the function of aquaporins in response to water stress as well as in regulating root water uptake. However, each PIP gene could play a specific role under specific circumstances (Aroca *et al.* 2011), and some studies have provided evidence to support this point. For example, overexpression of Arabidopsis PIP aquaporin gene *At*PIP1b in transgenic tobacco plant significantly increased plant growth rate and vigour, transpiration rate, as well as photosynthetic efficiency under favourable growth conditions but not under drought or salt stress conditions (Aharon *et al.* 2003). Similarly, under favourable growing conditions, overexpressing tobacco PIP aquaporin gene *Nt*AQP1 in Arabidopsis and tomato plants increased shoot growth, transpiration rate and photosynthetic efficiency (Sade *et al.* 2010). Conversely, antisense suppression of *Nt*AQP1 gene resulted in decreased root hydraulic conductivity and reduced water stress resistance but showed negligible modification in transpiration rate (Siefritz *et al.* 2002). Overexpression of a wheat PIP2 aquaporin gene *Ta*AQP7 increased drought tolerance in tobacco plants (Zhou *et al.* 2012), and likewise overexpression of tomato PIP genes *Sl*PIP2;1, *Sl*PIP2;7, and *Sl*PIP2;5 enhanced drought tolerance in tomato and Arabidopsis plants (Li R *et al.* 2016). In grapevine 'Brachetto', by overexpressing the *Vv*PIP2;4N gene (the most expressed PIP2 gene in root in Brachetto) in transgenic grape plants, Perrone *et al.* (2012) have concluded that *Vv*PIP2;4N had a substantial function in the regulation of root water relations under well-watered conditions but not under water-stressed conditions. Moreover, the authors suggested that other signals induced by water stress such as ABA might override the role of aquaporins and cause the lack of aquaporin-mediated regulation under water stress.

Taken together, the expression patterns of 7 PIP genes examined differ in the two grapevine rootstock varieties studied in our experiment under both well-watered and water-stressed conditions (see chapter 5). As concluded in Rossdeutsch *et al.* (2016) after comparing responses

to water deficit of 9 grapevine genotypes, responses to water deficit in grapevine are genotype-specific and closely associated with their genetic background. Moreover, it is difficult to draw clean lines between changes in the expression and function of PIPs in root water uptake as well in the regulation of root hydraulics. In the August-September 2016 experiment conducted on 110R, both root hydraulic conductivity (Figure 4.4) and *Vv*PIPs gene expression (panel C in Figure 4.7-4.13) were evaluated on the same plant. Based on what was discussed previously, decrease in root hydraulic conductivity in response to water stress (early stage) or decrease in root hydraulic conductivity in response to developmental stage (e.g., from early to mid stage) were not coupled with down-regulated *Vv*PIPs expression, but rather in an opposite direction. Perrone *et al.* (2012) have reported an inverse correlation between the expression level of *Vv*PIP2;4N (endogenous +transgene) and root hydraulic conductance. In general, it is assumed that up-regulated aquaporin expression level can improve plant's resistance to water stress due to its role in embolism reparation and possibly in inducing signals after changes in turgor pressure (Hill *et al.* 2004, Vandeleur 2007). When aquaporin expression and root hydraulics are down-regulated, it can prevent plant from losing water to drying soil. Concerning the contribution and regulation of aquaporins in root hydraulics, it is possible that other aquaporin genes are playing a critical role which is why we extended our expression studies to the entire MIP gene family via RNA-seq (see chapter 5). Hopefully we can find more information from the RNA-seq analysis. Martins *et al.* (2017) have reported that overexpression of a citrus aquaporin gene *Cs*TIP2;1 in transgenic tobacco plants increased plant growth under both optimal and stressed conditions and improved photosynthetic capacity, transpiration rate and water use efficiency of water-stressed plants. Furthermore, the regulation of aquaporins under well-watered and water-stressed conditions is not only restricted to the transcriptional level. Other approaches involved in aquaporin regulation include, e.g., trafficking to the membrane (Vera-Estrella *et al.* 2004), gating and subcellular trafficking mediated by phosphorylation, posttranslational modifications via phosphorylation, methylation, deamidation, NH_2-terminal acetylation, and ubiquitination, as well as heteromerization (see detailed review Maurel *et al.* 2015).

4.4.4 Relationship between root hydraulic conductivity/aquaporin gene expression and root growth rate

We observed some significant correlations between root hydraulic conductivity and root growth (only in 110R, Figure 4.14) as well as between the expression level of some *Vv*PIP genes and root growth rate (Figure 4.15-4.17). However, these correlations are quite noisy. For instance, a significant correlation between root hydraulic conductivity and root growth rate was found in 110R, but with a p-value of 0.047. The coefficient (r^2) observed for the correlation between *Vv*PIP gene expression and root growth rate was between 0.12 and 0.42.

In general, as discussed previously, water stress can affect both root water uptake (Lp_r) and root growth. Furthermore, according to Pritchard (1994), at the single cell level, root growth can be affected by turgor pressure and cell wall rheological properties. Turgor pressure is generated by the opposition of cell wall to water drawn into the cell. Then, wall-relaxation and decreasing

turgor caused by biochemical events within the wall will create a difference in water potential and allow water moving into the cell, thereby turgor is increased again. With water moving into the cell, turgor pressure expands the cell wall and the cell grows. Globally root growth is the sum of the individual cell expansions occurring along a file of cells in the apical regions of a root. Cell expansion could not occur without water entry (Pritchard 1994). Thus, we hypothesized that root growth could be tightly correlated with root water uptake which is normally evaluated as root hydraulic conductivity. However, the correlation between root water relations and root growth rate seems to be more complex. Potentially, with the results from our RNA-seq analysis, we could find genes that regulate root growth in addition to these *Vv*PIPs we analyzed. As reviewed in chapter 2, with the molecular advances in the study of root growth and development, our understanding of the mechanisms that control root development will be significantly improved. Internally, genes regulate root development and growth will be discovered and investigated; externally, mechanisms involved in root responses to environmental stimuli will be uncovered.

4.5 Concluding remarks

Changes in root hydraulic conductivity in response to plant developmental stages and water stress treatment are straightforward and consistent in both RGM and 110R. Root hydraulic conductivity decreased with progressed plant development under well-watered conditions and also decreased in response to water stress in the early stage during the period of the experiment. In our experiment, changes in root hydraulic conductivity did not correspond to traditionally defined differences in rootstock drought resistant capacity. Changes in PIPs gene expression varied depending on the specific gene, plant water status, developmental stages as well as genotype. Contribution of *Vv*PIPs to root water uptake and root hydraulics is hard to define and possibly other aquaporin genes as well as other complex regulation mechanisms are involved in controlling root water uptake in response to stress environment as well as along plant developmental stages.

Chapter 5 Short-term and long-term drought-induced transcriptomic changes in grapevine root aquaporins

Preliminary results from RNA-seq analysis

5.1 Introduction

Plasma membrane intrinsic proteins (PIPs) are generally considered as the most abundant aquaporins in root plasma membrane and play a central role in mediating transcellular root water uptake (Tournaire-Roux *et al.* 2003, Sutka *et al.* 2011). Thus, 7 *Vv*PIP genes were selected in our experiment to study their transcript abundances under both well-watered and water-stressed conditions in fine roots of these two rootstock varieties. However, aquaporins constitute a large family of membrane proteins, and apart from PIPs, there are several other subfamilies (e.g., TIPs and NIPs) with potential substantial physiological functions. A total of 35 aquaporin genes have been identified in the genomic sequence of Arabidopsis (Johanson *et al.* 2001), while 33 genes of aquaporins were identified in the genomic sequence of rice (Sakurai *et al.* 2005). In grapevine (*Vitis vinifera*), 23 full-length aquaporin genes have been previously identified by Shelden *et al.* (2009). Since then the original Pinot noir genome has been greatly improved and there has been a wealth of microarray and RNA-seq studies examining a huge breadth of circumstances in grapevine. Furthermore, new tools and approaches have been developed for analyzing the nature of genome duplications (Wang *et al.* 2012), as well as gene expression and cis-regulatory element structure (e.g., Wong *et al.* 2017). These improvements allow for a more comprehensive analysis of the grapevine MIP gene family (Wong *et al*. 2017 submitted).

In order to complete the analysis of aquaporin gene regulation on the transcriptional level, a more thorough transcriptomic analysis was performed by using RNA-seq technology on plants from different water stress levels and different developmental stages.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

Root samples analyzed in this chapter were from the experiments conducted in the 2016 growing season. RGM root samples were from the July-August experiment, and 110R root samples were from the August-September experiment. Concerning the plant materials and growth conditions as well as methods of the measurement of root growth and water potentials, they are identical as described in chapter 3. Briefly, one-year old dormant grapevine cuttings were planted in rhizotrons and kept in a greenhouse without supplementary lighting, temperature, or humidity control. The plants were watered until filed capacity right after plantation and were then

subjected to an automatic irrigation system with standard nutrient solution (Tandonnet *et al.* 2010). After an establishment period (usually around three weeks after the plantation), for each genotype, plants were randomly assigned to two treatments: well-watered conditions and water-stressed conditions. Plants under well-watered conditions were irrigated as during the establishment period, and plants under water-stressed conditions did not receive any water supply during the period of treatment. Root growth rate was measured by daily marking the position of root tips on the transparent paper pasted around rhizotron. $\Psi_{predawn}$ was determined in order to monitor the stress level of the plants.

5.2.2 Experimental design

Root samples of well-watered (CT), low level of water-stressed (WSL) as well as high level of water-stressed (WSH) plants were involved in the RNA-seq analysis. Given the method of water stress treatment used in our experiments, high level of water stress simultaneously means longer growing time. In order to take into account changes accompanied with plant growth and development, well-watered root samples were also harvested at the same time of sampling roots from high level water-stressed plants. Therefore, four categories of samples were acquired for each genotype: control low (CTL), WSL, control high (CTH), and WSH. Three biological replicates were designed for each category, making it a total of 24 samples for the two genotypes.

5.2.3 Total mRNA extraction

mRNA extraction was conducted as described in chapter 4. In brief, root tips of 5 cm long with known growth rate were harvested and frozen immediately in liquid nitrogen and kept in -80°C refrigerator until the time of analysis. Frozen samples were ground in liquid nitrogen into powder for RNA extraction. Total mRNA was extracted after Reid *et al.* (2006) and genomic DNA contamination was removed with the Turbo DNA-free kit (Life technologies, according to manufacturer's instructions).

5.2.4 RNA-sequencing

Total mRNA was sent to GeT-PlaGe Genome and Transcriptome platform (INRA Toulouse France) for RNA sequencing analysis. Ribosomal RNA depleted library construction was performed by GeT-PlaGe using their oprotocols and sequencing was performed on an Illumina HiSeq 2500 platform (Illumina) using paired-end generated reads. Fragments Per Kilobase of exon per Million (FPKM) mapped reads was calculated using edgeR.

5.2.5 Statistical analysis

Z-scores were calculated from FPKM values using the following equation:

$$z = (x - \mu) / \sigma$$

where x is the expression value, μ is the mean, and σ is the standard deviation across all samples. The Z score represents the deviation from the mean by standard deviation units.

For each genotype, the effects of drought treatment and developmental stage on the transcript abundance of each MIP gene (expressed in FPKM value) were analyzed using a two-way ANOVA ($p < 0.05$, with Tukey's HSD test).

5.3 Results

5.3.1 Plant water status

$\Psi_{predawn}$ of plants under different conditions in this experiment was plotted in Figure 5.1. The water status of well-watered plants was maintained at a high level during the period of the experiment (higher than -0.1 MPa). The low level water stress treatment decreased $\Psi_{predawn}$ (to around -0.2 MPa) for both genotypes but this decrease was statistically insignificant, while the high level water stress treatment significantly decreased $\Psi_{predawn}$ (to around -1.5 MPa).

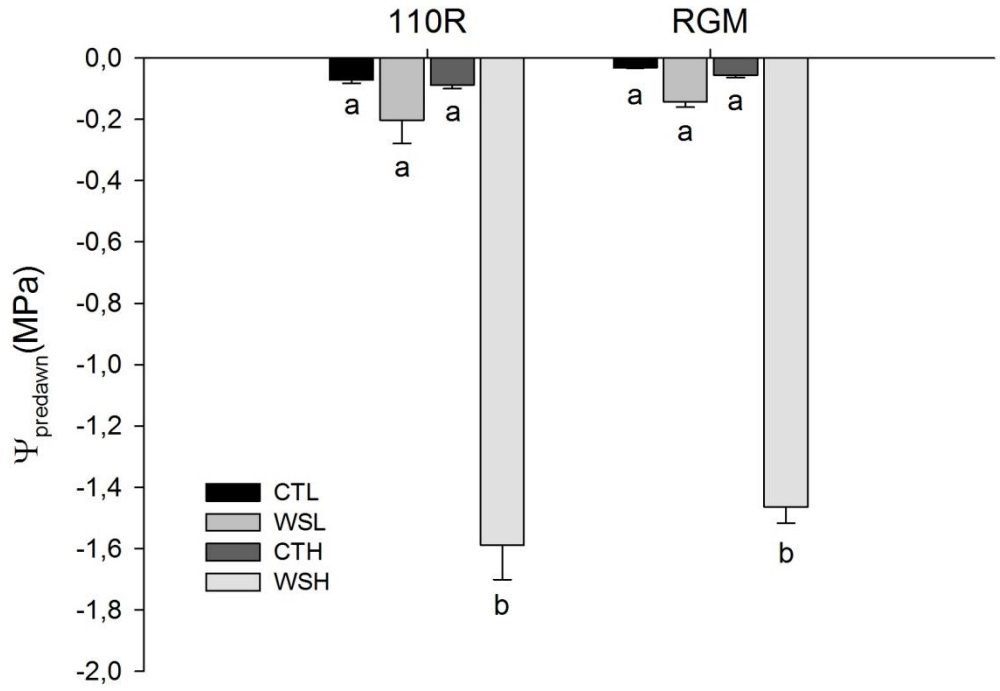

Figure 5.1 Pre-dawn leaf water potential for root samples analyzed with RNA-seq. Values are mean ± SE and different letters represent values that are significantly different (two-way ANOVA, Tukey's HSD test, $n = 3$, $p < 0.05$) within each genotype.

5.3.2 Genome-wide identification of MIP genes in grapevine

Following the work done by Shelden *et al.* (2009), in which 23 grapevine aquaporin genes were identified, Wong *et al.* (2017, submitted) further identified and annotated a total of 33 *Vitis vinifera* MIP family members including four truncated putative pseudogenes (*Vvi*PIP1-2b, *Vvi*PIP2-9, *Vvi*NIP9-1a and b) (Table 5.1). Phylogenetic tree and orthologous relationships between *Vitis vinifera* and Arabidopsis MIP families (Figure 5.2) were constructed (Wong *et al.* 2017, submitted). In our RNA-seq analysis, except for the four putative pseudogenes, the rest of the 29 MIP genes were all detected. The average expression levels of 29 MIP genes across all samples analyzed are illustrated in Figure 5.3, with *Vvi*PIP1-1, *Vvi*TIP2-1, *Vvi*PIP2-4, *Vvi*PIP1-3, *Vvi*TIP1-4, *Vvi*PIP2-7, and *Vvi*PIP2-5 being the 8 most abundantly expressed MIP genes in grapevine roots. However, the expression levels of seven MIP genes, including *Vvi*TIP5-2, *Vvi*PIP1-2c, *Vvi*NIP4-1, *Vvi*NIP7-1, *Vvi*TIP5-1, *Vvi*TIP3-1, and*Vvi*PIP3-1, were extremely low (FPKM values close or equal to 0). These seven genes were excluded from the subsequent analyses. The annotations of MIP genes used in our experiment are kept consistent with those from Wong *et al.* (2017, submitted). Moreover, according to the new annotations from Wong *et al.* (2017, submitted), the *Vvi*PIP2-1 and *Vvi*PIP2-2 genes studied in Gambetta *et al.* (2012) as well as in our previous RT-qPCR analysis are actually *Vvi*PIP2-5 and *Vvi*PIP2-7, respectively. *Vvi*PIP1-2-4 and *Vvi*PIP1-3-5 in our previous analysis correspond to *Vvi*PIP1-2a and *Vvi*PIP1-3, respectively, in the new version of annotation from Wong *et al.* (2017, submitted).

Table 5.1 List of grapevine aquaporin genes and detailed accession and homolog information

Gene Name	Protein Accession	Locus ID	Arapidopsis Homolog(s)
VviNIP1-2	VIT_10s0003g01830	Vitvi10g00639	AtNIP1-2 (AT4G18910)
VviNIP4-1	VIT_14s0006g01540	Vitvi14g00966	AtNIP4-1 (AT5G37810) or AtNIP4-2 (AT5G37820)
VviNIP5-1	VIT_02s0025g03260	Vitvi02g00295	AtNIP5-1 (AT4G10380) or AtNIP6-1 (AT1G80760)
VviNIP6-1	VIT_09s0070g00080	Vitvi09g00971	AtNIP5-1 (AT4G10380) or AtNIP6-1 (AT1G80760)
VviNIP7-1	VIT_05s0020g02740	Vitvi05g00432	AtNIP7-1 (AT3G06100)
VviNIP8-1	VIT_14s0108g00700	Vitvi14g01952	Ambiguous (low homology for all putative At homologues)
VviNIP9-1a	na	Vitvi14g00967	Ambiguous
VviNIP9-1b	na	Vitvi14g00968	Ambiguous
VviPIP1-1	VIT_13s0067g00220	Vitvi13g00012	Ambiguous
VviPIP1-2a	VIT_15s0046g02410	Vitvi15g01109	Ambiguous
VviPIP1-2b	na	Vitvi18g02210	Ambiguous
VviPIP1-2c	VIT_12s0034g00250	Vitvi12g01740	Ambiguous
VviPIP1-3	VIT_02s0025g03390	Vitvi02g00310	Ambiguous
VviPIP1-4	VIT_15s0046g02420	Vitvi15g01110	Ambiguous
VviPIP2-3	VIT_08s0040g01890	Vitvi08g01038	Ambiguous
VviPIP2-4	VIT_06s0004g02850	Vitvi06g00281	Ambiguous
VviPIP2-5	VIT_13s0019g04280	Vitvi13g00605	Ambiguous
VviPIP2-7	VIT_03s0038g02520	Vitvi03g00155	AtPIP2-7 (AT4G35100) or AtPIP2-8 (AT2G16850)
VviPIP2-9	na	Vitvi10g00803	Ambiguous
VviPIP3-1	VIT_03s0038g01390	Vitvi03g00081	Ambiguous
VviPIP3-2	VIT_03s0038g01410	Vitvi03g00083	Ambiguous
VviSIP2-1	VIT_08s0040g00400	Vitvi08g00904	AtSIP2-1 (AT3G56950)
VviTIP1-1	VIT_06s0061g00730	Vitvi06g01346	Ambiguous
VviTIP1-2	VIT_08s0007g04780	Vitvi08g01602	Ambiguous
VviTIP1-3	VIT_13s0019g00330	Vitvi13g00255	AtTIP1-3 (AT4G01470)
VviTIP1-4	VIT_06s0004g04120	Vitvi06g00412	Ambiguous
VviTIP2-1	VIT_09s0002g04020	Vitvi09g00329	AtTIP2-1 (AT3G16240)
VviTIP2-2	VIT_00s2783g00010	Vitvi00g01417	Ambiguous
VviTIP2-3	VIT_00s0229g00130	Vitvi02g00568	AtTIP2-3 (AT5G47450)
VviTIP3-1	VIT_16s0039g00220	Vitvi16g00010	AtTIP3-1(AT1G73190) or AtTIP3-2 (AT1G17810)
VviTIP4-1	VIT_04s0008g03550	Vitvi04g00307	AtTIP4-1 (AT2G25810)
VviTIP5-1	VIT_16s0022g00330	Vitvi16g00655	AtTIP5-1 (AT3G47440)
VviTIP5-2	VIT_15s0021g02420	Vitvi15g00629	Ambiguous

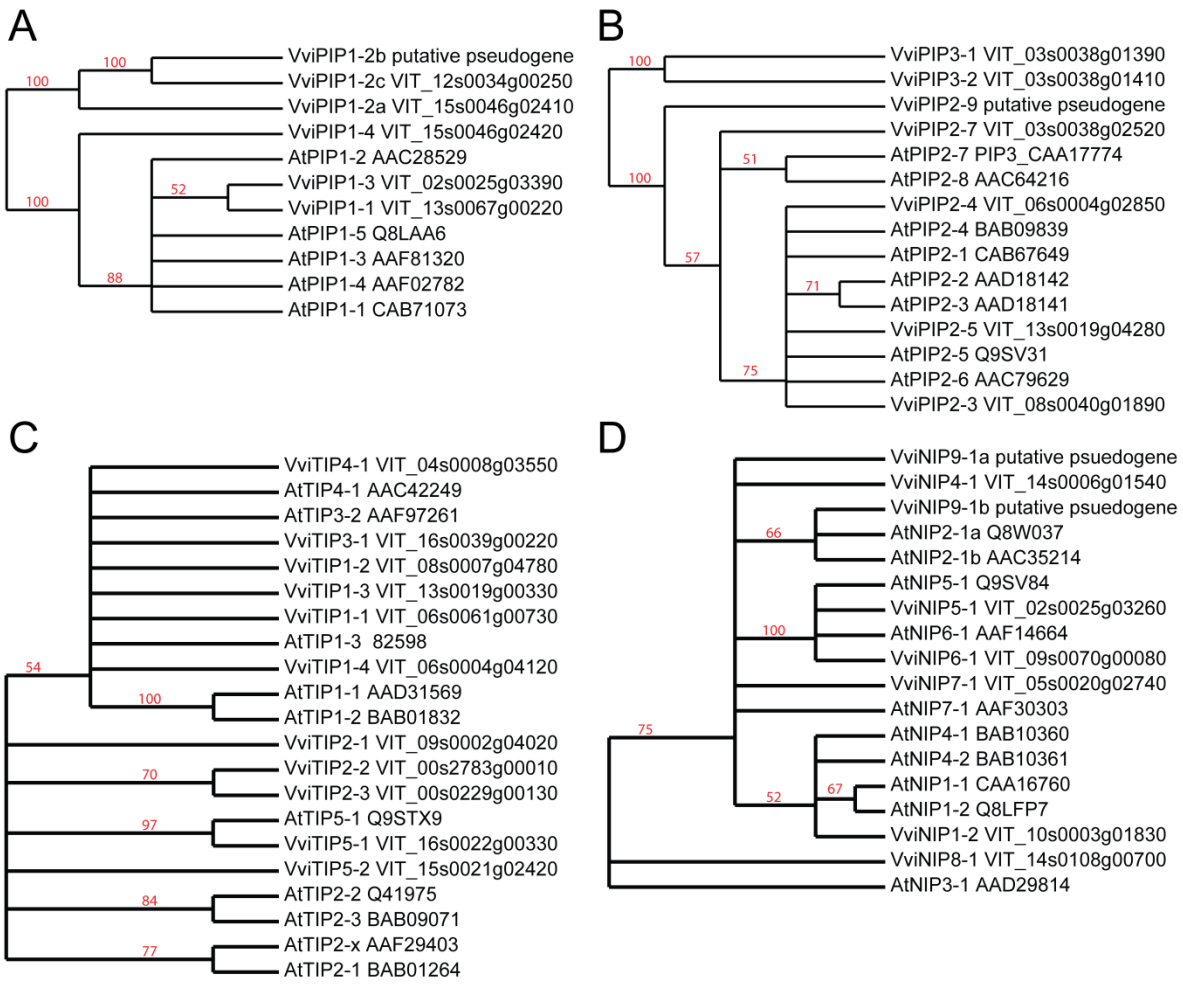

Figure 5.2 Protein sequence relationships between the *Vitis vinifera* and Arabidopsis MIP families. The four major MIP sub-families: PIP1s (A), PIP2s (B), TIPs (C), and NIPs (D). Red numbers represent bootstrap values and the tree was collapsed for all bootstrap values under 50 (100 bootstrap replicates). *Vvi*SIP2-1 was not included in this analysis. Detailed accession and homology information is presented in Table 5.1.

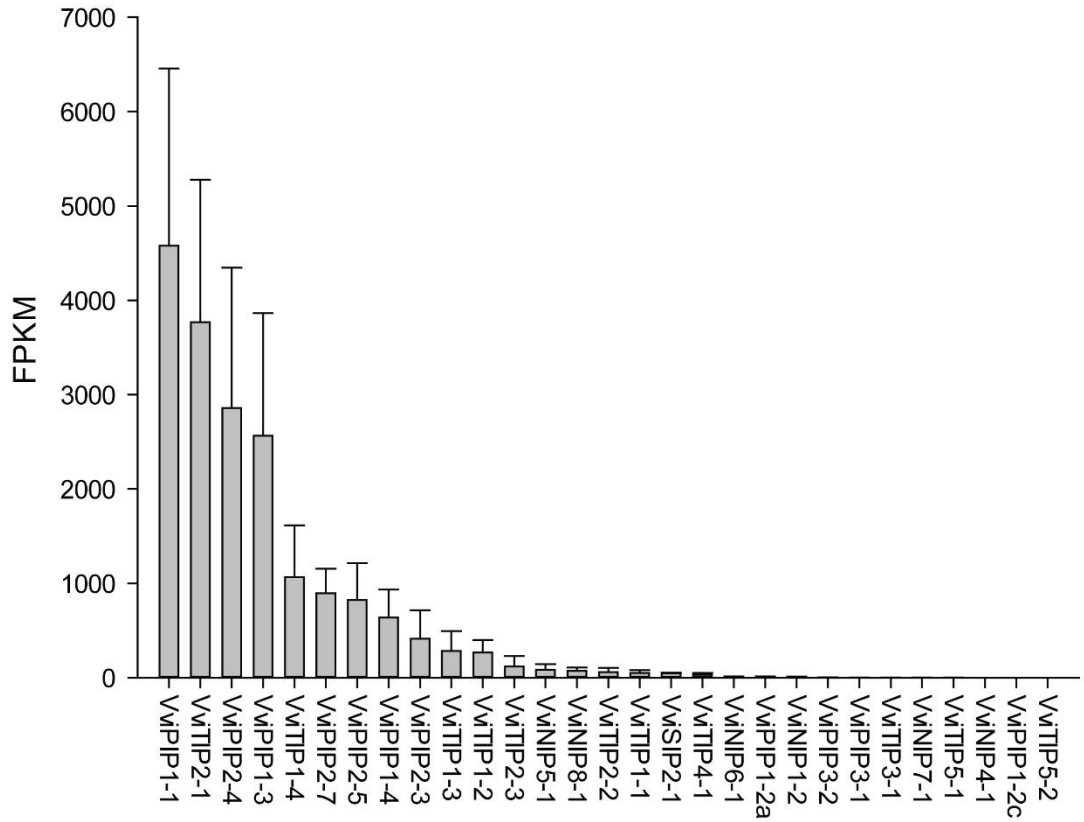

Figure 5.3 Global FPKM averages of 29 MIP genes detected in our RNA-seq analysis. Values are mean ± SE.

Table 5.2 Seven most abundantly expressed MIP genes in each group from high to low level

Transcript Abundance	110R CTL	110R WSL	110R CTH	110R WSH	RGM CTL	RGM WSL	RGM CTH	RGM WSH
1	*Vvi*PIP1-1	*Vvi*PIP1-1	*Vvi*TIP2-1	*Vvi*PIP1-1	*Vvi*TIP2-1	*Vvi*PIP1-1	*Vvi*PIP1-1	*Vvi*PIP1-1
2	*Vvi*TIP2-1	*Vvi*TIP2-1	*Vvi*PIP1-1	*Vvi*PIP1-3	*Vvi*PIP1-1	*Vvi*PIP2-4	*Vvi*TIP2-1	*Vvi*PIP1-3
3	*Vvi*PIP2-4	*Vvi*PIP2-4	*Vvi*PIP2-4	*Vvi*PIP2-4	*Vvi*PIP1-3	*Vvi*TIP2-1	*Vvi*PIP2-4	*Vvi*PIP2-4
4	*Vvi*TIP1-4	*Vvi*PIP1-3	*Vvi*PIP1-3	*Vvi*TIP2-1	*Vvi*PIP2-4	*Vvi*PIP1-3	*Vvi*PIP1-3	*Vvi*TIP2-1
5	*Vvi*PIP1-3	*Vvi*PIP2-5	*Vvi*TIP1-4	*Vvi*PIP2-7	*Vvi*TIP1-4	*Vvi*TIP1-4	*Vvi*TIP1-4	*Vvi*PIP2-7
6	*Vvi*PIP2-5	*Vvi*PIP1-4	*Vvi*PIP1-4	*Vvi*PIP1-4	*Vvi*PIP2-7	*Vvi*PIP2-7	*Vvi*PIP2-7	*Vvi*PIP2-5
7	*Vvi*PIP2-7	*Vvi*TIP1-4	*Vvi*PIP2-5	*Vvi*PIP2-5	*Vvi*PIP2-5	*Vvi*PIP2-5	*Vvi*PIP2-5	*Vvi*TIP1-4

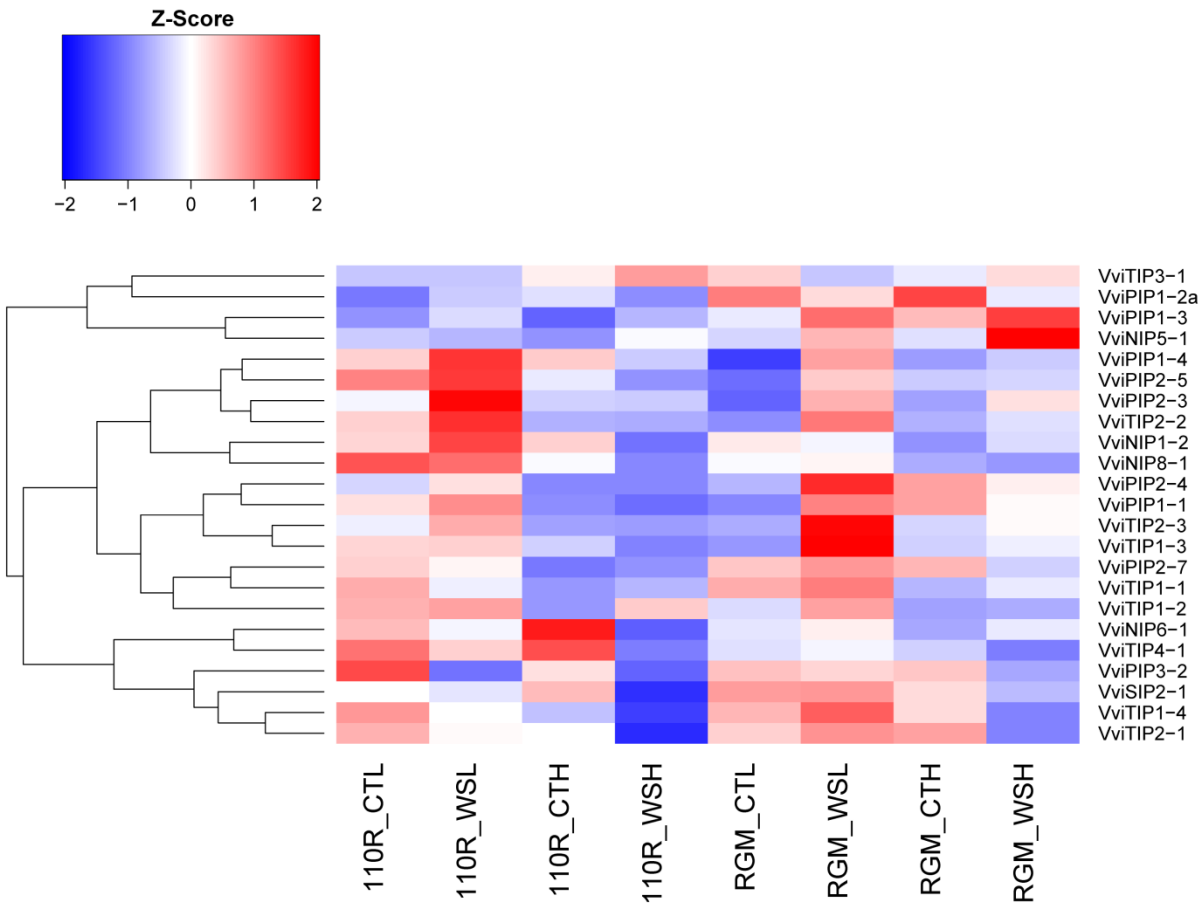

Figure 5.4 Heatmap of normalized FPKM data from 110R and RGM under low and high levels of water stress (WSL and WSH) treatments as well as their corresponding controls (CTL and CTH).

5.3.3 Transcriptomic changes of MIP genes in response to water stress and developmental stages in grapevine root

Very diverse expression patterns for the 22 MIP genes analyzed in our experiment were observed in the transcriptome data representing different genotypes, different water stress treatments, as well as different developmental stages. For most MIP genes within each genotype, the expression levels were influenced either by water stress treatment, or by developmental stage, or by both factors. In correspondence with the average expression levels of the 29 MIP genes across all samples illustrated in Figure 5.3, in each group of the analyzed samples, there were always seven out of the eight most abundantly expressed MIP genes across all samples analyzed that are the most expressed by treatment category as presented in Table 5.2. The heatmap of z-score for all the samples analyzed are presented in Figure 5.4. For 110R, under low level of water stress treatment, three genes, *Vvi*PIP1-4, *Vvi*PIP2-3, and *Vvi*TIP2-2, were significantly up-regulated, while one gene, *Vvi*PIP3-2, was significantly down-regulated; under high level of water stress

treatment, one gene, *Vvi*NIP5-1, was significantly up-regulated, while six genes, *Vvi*NIP6-1, *Vvi*TIP4-1, *Vvi*PIP3-2, *Vvi*SIP2-1, *Vvi*TIP1-4, and *Vvi*TIP2-1, were significantly down-regulated. In well-watered plants, three *Vvi*TIP genes, *Vvi*TIP2-2, *Vvi*TIP1-3, and *Vvi*TIP1-4, were significantly down-regulated in late developmental stage compared with early developmental stage. For RGM, under low level of water stress, 8 genes, *Vvi*PIP1-1, *Vvi*PIP2-4, *Vvi*PIP2-5, *Vvi*PIP1-4, *Vvi*PIP2-3, *Vvi*TIP1-3, *Vvi*TIP2-3, and *Vvi*TIP2-2, were significantly up-regulated, while the expression level of other MIP genes did not show significant differences; under high level of water stress, one gene, *Vvi*NIP5-1, was significantly up-regulated, while five genes, *Vvi*TIP2-1, *Vvi*TIP1-4, *Vvi*TIP4-1, *Vvi*SIP2-1, and *Vvi*PIP3-2, were significantly down-regulated. In well-watered plants, no significant differences in MIP gene expression were observed in late developmental stage compared with early developmental stage. Taken together, the patterns of changes in the expression levels of MIP genes in grapevine root in response to water stress and developmental stage differ between rootstock genotypes. For instance, in well-watered plants, the expression level of MIP genes in RGM did not show drastic regulations over development, while three *Vvi*TIPs were significantly down-regulated in late developmental stage in 110R. In plants subjected to low level of water stress treatment, three identical genes were all significantly up-regulated in both RGM and 110R; however, 5 more MIP genes from *Vvi*PIP and *Vvi*TIP subfamilies were up-regulated in RGM. Nevertheless, for both genotypes, more genes were up-regulated under low level of water stress treatment, while in contrast, more genes were down-regulated under higher level of water stress treatment. Under low level of water stress, in addition to *Vvi*PIP1-4, *Vvi*PIP2-3, and *Vvi*TIP2-2, five more genes were significantly up-regulated in RGM in comparison to 110R; no genes were significantly down-regulated in RGM, while one gene was significantly down-regulated in 110R. Under high level of water stress, the only gene that was significantly up-regulated in both genotypes was *Vvi*NIP5-1, and all significantly down-regulated five MIP genes in RGM were consistent with those in 110R, except for *Vvi*NIP6-1, which was the 6th most down-regulated MIP gene in 110R. Four MIP genes were significantly down-regulated in response to developmental stage in 110R under well-watered conditions, while no significant regulation in terms of the transcript abundances in MIP genes was observed in RGM.

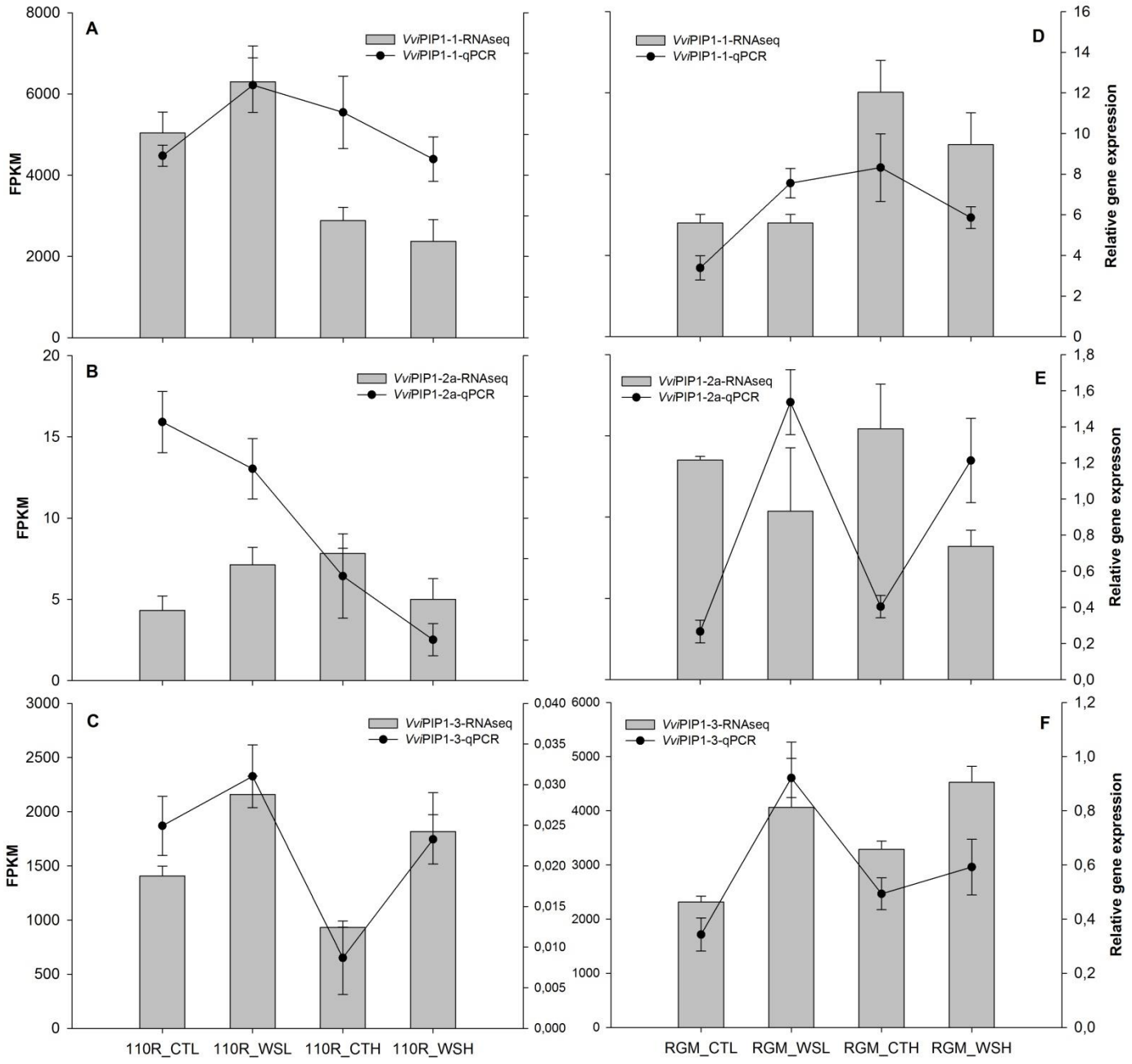

Figure 5.5 Comparison of gene expression levels of *Vvi*PIP1s between RNA-seq and RT-qPCR

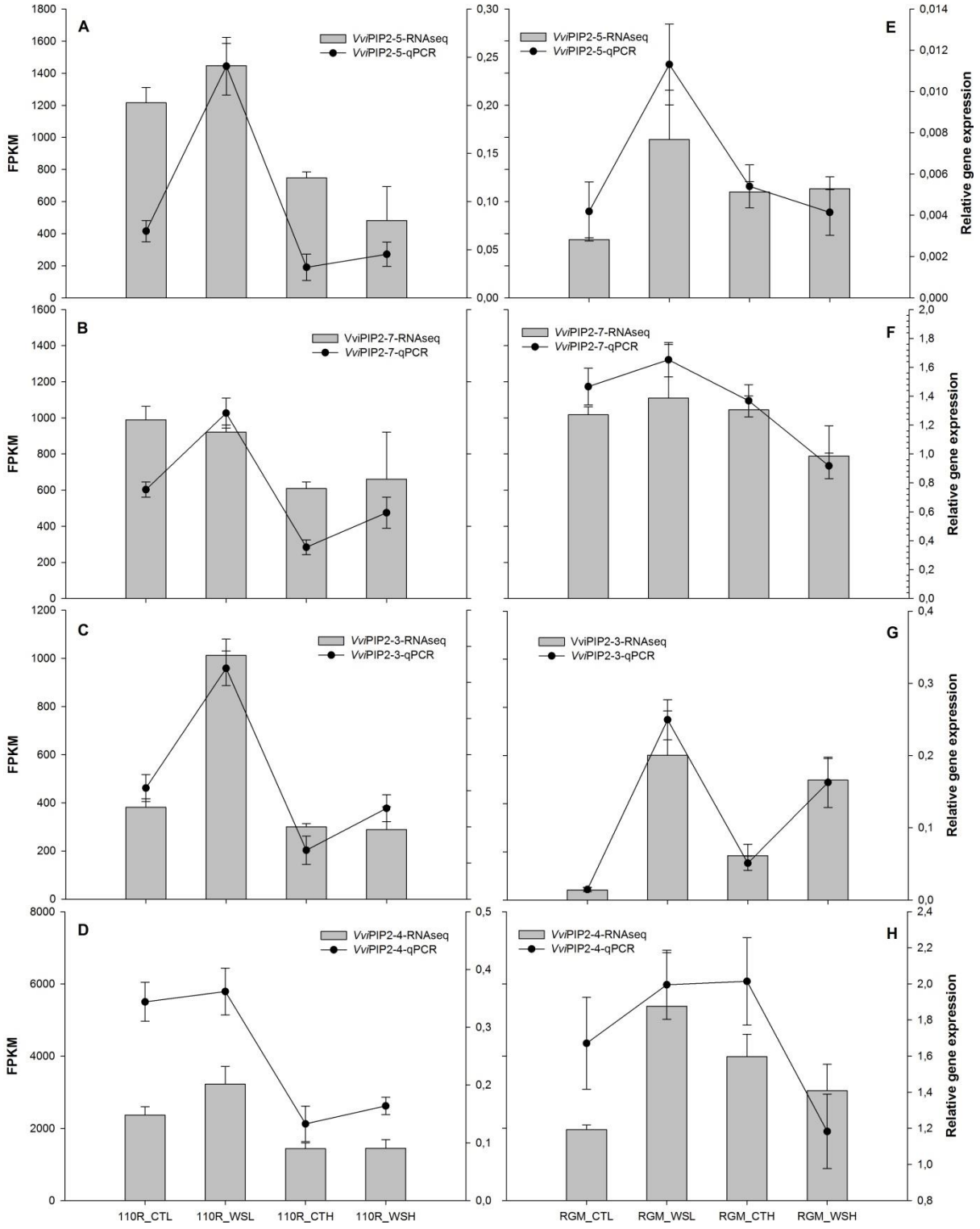

Figure 5.6 Comparison of gene expression levels of *Vvi*PIP2s between RNA-seq and RT-qPCR

5.3.4 Comparison of RT-qPCR results with RNA-seq results

For both genotypes, transcript abundances of seven *Vvi*PIP genes previously analyzed with RT-qPCR method were plotted together (Figure 5.5 and 5.6) with those from RNA-seq analysis in order to compare the consistency of MIP gene expression patterns measured with two different methodologies. For 110R, of the seven *Vvi*PIP genes analyzed by RT-qPCR, two *Vvi*PIP1 genes, *Vvi*PIP1-1 and *Vvi*PIP1-3, and three *Vvi*PIP2 genes, *Vvi*PIP2-5, *Vvi*PIP2-3, and *Vvi*PIP2-4, exhibited similar changes in their expression patterns under various conditions with those identified by RNA-seq analysis. For RGM, one *Vvi*PIP1 gene, *Vvi*PIP1-3, and three *Vvi*PIP2 genes, *Vvi*PIP2-5, *Vvi*PIP2-7, and *Vvi*PIP2-3, were observed to have similar tendency in changes in the expression patterns when analyzed by both methods. Regardless of the similarity in changes in the expression patterns under different conditions determined by RT-qPCR and RNA-seq, the relative magnitudes of gene expression levels of some genes exhibited some inconsistency between these two analysis approaches. For both genotypes, *Vvi*PIP1-1 was the most expressed PIP gene detected in our experiment by both RT-qPCR and RNA-seq. Results from RNA-seq showed that for both genotypes, *Vvi*PIP1-2a was the least expressed PIP genes among three PIP genes selected with the magnitude of the expression level being 1/400 of that of *Vvi*PIP1-1, while *Vvi*PIP1-3 was the second most expressed PIP gene with the magnitude of the expression level being 1/3 of that of *Vvi*PIP1-1 in 110R and 2/3 in RGM. In contrast, for both genotypes, *Vvi*PIP1-2a was the second most expressed PIP genes according to the results from RT-qPCR with the magnitude of the expression level being 1/10 of that of *Vvi*PIP1-1. *Vvi*PIP1-3 was the least expressed PIP genes for both genotypes determined with RT-qPCR but showed a huge difference in terms of the expression level between these two genotypes. In RGM, the magnitude of the expression level of *Vvi*PIP1-3 was 1/15 of that of *Vvi*PIP1-1, while in 110R, the value was 1/400 of that of *Vvi*PIP1-1. Differences concerning the magnitude of the expression levels were observed in the four selected *Vvi*PIP2 genes as well. Overall, the relative expression levels of *Vvi*PIP2 genes determined with RT-qPCR were much lower than those from RNA-seq analysis. The relative expression levels within the four *Vvi*PIP2 genes also differed between the results obtained from these two methods.

5.4 Discussions

In order to obtain a better understanding of the molecular mechanisms involved in grapevine responses to water stress, RNA-seq analysis was conducted to investigate the global transcriptome changes in root tissue. Regulation of aquaporins during plant adaptation to water stress is of particular interest in our experiment. Thus, modifications of the expression profiles of the MIP gene family was discussed in detail in this chapter. A total of 29 MIP genes were identified in our study. However, the expression levels of seven genes were extremely low. Therefore, only the remaining 22 MIP genes were further analyzed and discussed.

For both genotypes studied in our experiment, significant changes in the transcript abundances of various MIP genes were detected under both low and high water stress levels. Noteworthily, we

observed that more MIP genes were up-regulated in plants subjected to low level of water stress, while more genes were down-regulated in plants subjected to high level of water stress. Generally, as indicated in the previous chapter, PIP aquaporin genes are considered to play a critical role in plasma membrane and transcellular water transport in roots. A total of 11 *Vvi*PIP genes excluding two putative pseudogenes, *Vvi*PIP1-2b and *Vvi*PIP2-9, were identified in our experiment with five PIP1 genes and four PIP2 genes (Table 5.1), of which seven were abundantly expressed across all samples analyzed and constituted nine most expressed MIPs in grapevine roots together with another two *Vvi*TIPs, *Vvi*TIP2-1 and 1-4 (Figure 5.3). In addition to their transcript abundancy, certain PIPs also played a central role in response to water stress. For example, *Vvi*PIP1-4 and 2-3 were significantly up-regulated in both 110R and RGM under low level of water stress, and *Vvi*PIP3-2 was significantly down-regulated in both 110R and RGM under high level of water stress.

Aside from *Vvi*PIPs, *Vvi*TIPs were observed to have important expression levels as well in our experiment and were also involved in the regulation of aquaporin in response to different levels of water stress. A total of 11 *Vvi*TIPs were identified in our study, in which three of them, *Vvi*TIP3-1, 5-1, and 5-2, were extremely low expressed and were not considered in the discussion. Consistent with our observations, TIPs have been reported to show high expression levels in root tissues in many other species (Maurel *et al.* 2015). For both 110R and RGM, *Vvi*TIP2-2 was significantly up-regulated under low level of water stress, while *Vvi*TIP2-1, 1-4, and 4-1 were significantly down-regulated under high level of water stress. Moreover, drastic down-regulation of MIP genes was observed in 110R related to plant developmental stage, and all the down-regulated genes belong to the *Vvi*TIP subfamily (*Vvi*TIP1-4, 1-3, and 2-2). Similar to PIPs, who facilitate transcellular water transport towards expanding tissues, TIPs also seem to play a critical role in plant growth (Maurel *et al.* 2015). For example, in Arabidopsis, the expression of γ-TIP (*At*TIP1-1) has been reported to be associated with cell enlargement in the elongation zone in root tips (Ludevid *et al.* 1992). In tomato (*Solanum lycopersicum*), *SI*TIP2-2 was induced in response to abiotic stress (Sade *et al.* 2009), which is in agreement with our observation. Furthermore, it has been reported that constitutive expression of *Sl*TIP2-2 in transgenic tomato plants increased cell osmotic water permeability and whole-plant transpiration and improved plant growth performance under both normal and water-stressed conditions by favoring their anisohydric behavior (Sade *et al.* 2009). Therefore, the authors concluded that whole-plant tolerance to abiotic stress might depend on the regulation mechanisms controlling tonoplast water permeability (Sade *et al.* 2009). Overexpression of a of Ginseng (*Panax ginseng*) TIP ortholog (*Pg*TIP1) in transgenic Arabidopsis plants significantly increased overall plant growth through increased growth and development of plant cells under favorable conditions (Lin *et al.* 2007, Peng *et al.* 2007). Under water-stressed conditions, the transgenic plants were significantly more tolerant to water stress when grown in deeper pots, which could result from changes in root morphology and leaf water channel activity in transgenic plants (Peng *et al.* 2007). It has been suggested that TIPs might play a critical role in osmoregulation and vacuolar differentiation in expanding cells (Maurel *et al.* 2015). In grapevine, the expression of *Vvi*TIP2-1 has been

observed to be closely correlated with leaf hydraulic and stomatal conductances under both well-watered and water-stressed conditions, which could possibly be a signal involved in leaf hydraulic control (Pou *et al.* 2013).

Our previous analysis has focused on PIPs; however, from the results of the RNA-seq analysis, TIPs seem to play an important role in the regulation of plant water status as well. Moreover, TIPs seem to have a much higher lateral membrane mobility due to a higher fluidity of vacuolar membrane in comparison to plasma membrane (Luu *et al.* 2012, Hosy *et al.* 2015). The name of TIPs appears to indicate the specific localization of TIP aquaporin proteins. However, TIPs have been reported of dual subcellular localizations and are presented in both vacuolar membrane and plasma membrane (Maurel *et al.* 2015).

Overall, from the aquaporin gene expression data obtained in our RNA-seq analysis, PIPs and TIPs seem to be two MIP subfamilies most involved in the molecular regulation of plant water-related behavior. Generally, studies in root aquaporin expression have focused on PIPs and TIPs (Maurel *et al.* 2015). A strong correlation between PIP and TIP aquaporin expression and cell expansion has been observed in different plant materials (Maurel *et al.* 2008). Most of the PIPs and TIPs show high water permeability and function as efficient water channels. In addition, they also facilitate the transport of other small substrates such as H_2O_2 and CO_2 for PIPs, and NH_3 and urea for TIPs (Gerbeau *et al.* 1999, Jahn *et al.* 2004, Loqué *et al.* 2005, Bienert and Chaumont 2014).

A total of six *Vvi*NIPs were identified in our study, two of which showed extremely low transcript levels and were not included in the analysis. NIPs are a subfamily of aquaporin proteins called the Nodulin-26 like intrinsic proteins and can be divided into three subgroups based on their distinctive structure characterized by substitutions within the aromatic arginine (ar/R) selectivity filter (Mitani *et al.* 2008, Beamer *et al.* 2015). NIPs show a broad range of subcellular localization patterns, for example, *At*NIP2-1 is localized in the endoplasmic reticulum and the plasma membrane, and *Os*NIP2-1 and *At*NIP5-1 are localized in the plasma membrane (Maurel *et al.* 2008). In contrast to PIPs and TIPs, all NIPs investigated tend to have a reduced water transport activity and instead exhibited high permeability to small organic solutes and mineral nutrients (Ma *et al.* 2006, Takano *et al.* 2006) participating in a number of osmoregulatory and metabolic functions (Beamer *et al.* 2015). In particular, they mediate the transport of beneficial (e.g., boron, silicon, selenium) or toxic (e.g. arsenic, antimony) metalloids (Maurel *et al.* 2015), for instance, three Arabidopsis NIPs, *At*NIP2-1 *At*NIP5-1, and *At*NIP7-1, have been reported to facilitate the transport of silicic acid, boric acid, and arsenic acid, respectively (Ma *et al.* 2006, Takano *et al.* 2006, Isayenkov *et al.* 2008). Apart from being the most expressed *Vvi*NIP gene, *Vvi*NIP5-1 is also the only MIP gene that was significantly up-regulated under high level of water stress treatment for both genotypes in our experiment. As illustrated in Figure 5.2, *Vvi*NIP5-1 is homologue to *At*NIP5-1 and *At*NIP6-1 which are both characterized by a lack of water transport activity and are essential transporters for boric acid (Takano *et al.* 2006, Tanaka *et al.* 2008). Boron (B) is known to be an essential micronutrient for higher plants and the importance of B to

plants' growth and development has been widely acknowledged (e.g., Dell and Huang 1997, Blevins and Lukaszewski 1998, Bariya *et al.* 2014, Durbak *et al.* 2014). B has been reported to play an important role in root elongation (Kouchi and Kumazawa 1975) and is also crucial for the maintenance of cell wall organization and properties (O'Neill *et al.* 2004, Takano *et al.* 2006, Durbak *et al.* 2014). B has also been suggested to be involved in the plasma membrane transport processes, as well as in membrane integrity by cross-linking the membrane molecules containing hydroxlated ligands such as glycoproteins and glycolipids (Goldbach *et al.* 2001, Wimmer *et al.* 2009, Bariya *et al.* 2014). Changes in membrane potential in *Daucus carota* under B deficiency have been reported (Blaser-Grill *et al.* 1989). Moreover, limited B has been observed to modify the permeability of plasma membrane to ions and other solutes (Wang *et al.* 1999, Carmen Rodríguez-Hernández *et al.* 2013). The up-regulation of *Vvi*NIP5-1 under high water stress level is potentially linked with increased B uptake in grapevine root, which could possibly help to maintain root growth, protect root cell wall structure and function, and favor the uptake of water as well as other ions/nutrients, particularly under severe water stress. In addition to NIP5-1, *Os*NIP3-1 in rice (*Oryza sativa* L.) has also been discovered to function as a boric acid channel and contribute to the regulation of boron distribution among shoot tissues as correct boron distribution is crucial for plant growth (Hanaoka *et al.* 2014). Moreover, under high level of water stress, the up-regulation of *Vvi*NIP5-1, encoding an aquaporin with low water transport activity, associated with the down-regulation of several MIP genes encoding aquaporins with high water channel activity, can be potentially a mechanism to prevent water loss back to soil as previously discussed in chapter 4.

*Vvi*SIP2-1 is the only SIP gene detected in our experiment. SIPs are small intrinsic protein located at the endoplasmic reticulum (ER), but not at the plasma or vacuolar membranes (Ishikawa *et al.* 2005). Two SIP1s and one SIP2 have been identified and functionally characterized in Arabidopsis (Ishikawa *et al.* 2005). Two SIP1s showed water channel activity but not SIP2 (Ishikawa *et al.* 2005). In grapevine, *Vvi*SIP1 was found to express in leaves, berries and stems, but not in roots (Noronha *et al.* 2013), which is consistent with our observation. In our experiment, *Vvi*SIP2-1 was significantly down-regulated under high level of water stress for both genotypes. However, not a lot of studies have been focused on SIPs, so their mode of function in ER is not clear yet (Maurel *et al.* 2015). Promisingly, a deeper investigation on SIPs, aquaporins that are confined in the ER, may reveal novel aspects of plant cell osmoregulation (Ishikawa *et al.* 2005, Noronha *et al.* 2013, Maurel *et al.* 2015).

110R and RGM are two grapevine rootstock varieties with inherently different drought resistant capacity. As indicated in the beginning of the thesis, 110R is considered to be highly resistant to drought stress while RGM is sensitive to drought stress (Carbonneau 1985). Due to their different genetic background, transcript abundances of MIP genes differed in 110R and RGM under well-watered conditions, and so did changes in MIP transcripts in their responses to different levels of water stress treatment. Under well-watered conditions, three *Vvi*TIPs, *Vvi*TIP1-3, 1-4, and 2-2, were significantly down-regulated in 110R over development, while no drastic changes in terms

of the expression levels of MIPs were observed in RGM. Under low level of water stress, one MIP gene, *Vvi*PIP3-2, was significantly down-regulated in 110R, while no MIP genes were significantly down-regulated in RGM. On the contrary, four more genes, *Vvi*PIP1-1, 2-4, 2-5, and *Vvi*TIP2-3 were significantly up-regulated in RGM compared to 110R. Aquaporin expression patterns can serve as a useful indicator for the contribution of water channels to root water transport (Maurel *et al.* 2015). Moreover, as already discussed earlier in chapter 4 regarding PIP genes, each PIP gene could play a specific role under specific circumstances (Aroca *et al.* 2011). Therefore, different behaviors exhibited in plant water relations in these two varieties can be attributed to variances in their aquaporin expression patterns. In rice (*Oryza sativa* L.), it has been reported that differences in aquaporin gene regulation possibly contributed to distinct adaptive mechanisms to water deficit (Lian *et al.* 2004). In grapevine cultivars that differ in drought tolerance, Vandeleur *et al.* (2009) have revealed that *Vvi*PIP1-1 was up-regulated in the cultivar which is less tolerant to drought, while it remained constant in the cultivar which is more tolerant to drought.

Concerning the relevance for the results of mRNA expression level between RT-qPCR and RNA-seq analysis, overall, a majority of genes determined with RT-qPCR showed similar expression manners as identified using RNA-seq. However, the relative expression levels between seven selected genes differed to a large content comparing RT-qPCR and RNA-seq analysis. The RT-qPCR analysis used in our experiment to evaluate gene expression level is actually a relative quantification method. Therefore, the results could largely depend on the different reference genes used. On the contrary, RNA-seq analysis is an absolute quantification technique. In this sense, RNA-seq analysis can reveal more accurate results, and thus offer more insights in mechanisms involved in plant response to water stress on a molecular level.

5.5 Concluding remarks

RNA-seq analysis complemented the RT-qPCR analysis on seven selected *Vvi*PIP genes resulting in a more comprehensive understanding in grapevine responses to water stress on a transcriptional level. We consistently observed that for both 110R and RGM more genes were up-regulated under low level of water stress while more genes were down-regulated under high level of water stress. Differentially expressed MIP genes differed in these two genotypes. However, consistent regulation of certain MIP genes under water stress was observed in 110R and RGM. Some less studied MIP genes such as NIPs and SIPs seem to contribute to the regulations in response to water stress as well. But the mechanisms are still unknown. Comparison of gene expression data from RT-qPCR and RNA-seq revealed that there is a good correspondence in gene expression patterns for the majority of genes between these two methods.

Chapter 6 General discussions and conclusions

In the context of global climate change, there is increasing focus and demand for more drought resistant plant material. Grapevine is a widely cultivated and economically important crop. However, markets often dictate specific grape varieties that can be grown and sold. Thus, growers are increasingly interested in conferring particular traits of interest (e.g., drought tolerance) through grafting onto rootstocks (Zhang *et al*. 2016). In this sense, much focus has been placed on the understanding of rootstocks effects on scion growth, nutrient uptake, and tolerance to stress, with the ultimate goal of developing novel rootstocks that facilitate adaptation to a changing climate. Based on previous findings, it is suggested that root water uptake could be tightly coupled to a root's instantaneous rate of growth (see Gambetta *et al.* 2013), which implies that differences in drought resistance between genotypes could result largely from their ability to maintain root growth under stress. Therefore, the original aim of these experiments was to determine the relationship between root growth rate and root water uptake in two grapevine rootstocks with contrasting drought resistant capacity, under well-watered and different levels of water-stressed conditions. Whether root growth and root water uptake are related to changes in the expression levels of aquaporin genes was investigated as well.

Root growth

Prolonged water stress treatment decreased plant water potential. Individual root growth rate is very heterogeneous, although drought treatment reduces root elongation rate on average, individual root growth rate still varies enormously. The dynamics of root growth are the result of the interaction between the internal growth mechanisms and the external impacts of environmental conditions (Walter and Schurr 2005, Walter *et al.* 2009). Changes in root growth rate in response to water stress depend largely on the degree of water stress. However, the exact mechanisms behind the responses of root system to water stress are not completely clear. Maintenance of root elongation rate under water stress has been reported by many researchers (e.g., Sharp *et al.* 1988, Spollen *et al.* 1993, Sharp *et al.* 2004), and evidence has been shown that this maintenance is kept preferentially towards root apex (Sharp *et al*. 2004). In contrast, elongation rate of more basal zones along the length of the root is more inhibited, as a result, the length of the growing zone along the root was shortened with decreased water potential (Sharp *et al*. 1988). Stimulated root growth under water stress has been observed as well (Rodrigues *et al.* 1995). Sustained root growth under drought has been considered as an adaptive response which is related with osmotic adjustment and an enhanced cell wall loosening capacity (Saab *et al.* 1992, Hsiao and Xu 2000, Chaves *et al.* 2002, Sharp *et al.* 2004). Plant hormones such as ABA may also be involved in plants' responses to water stress.

In general, high level of water stress treatment significantly reduced average root growth rate for both RGM and 110R. RGM and 110R did not show any differences in terms of changes in root

growth rate in response to water stress. What is noteworthy is that, globally, regardless of water stress treatment, average root growth rate showed a decreased trend over plant development in both genotypes.

Under well-watered conditions, higher root growth rates were constantly observed in 110R compared to RGM, which could be one possible explanation for the higher capacity in drought resistance of 110R.

Soil temperature is also a factor that affects root growth. For both RGM and 110R, under both well-watered and water-stressed conditions, average daily root growth rate was positively correlated with average daily soil temperature. However, the correlation was weak and noisy.

Root hydraulic conductivity

RGM and 110R did not respond differently to water stress in terms of changes in root hydraulic conductivity, which suggested that Lp_r may not be a good indicator for rootstock drought resistance.

Root hydraulic conductivity was influenced by both water stress treatment and plant developmental stage. Generally, for both RGM and 110R, Lp_r was significantly reduced under water stress in early stage. In mid and late stages, no significant differences in Lp_r were observed between well-watered and water-stressed plants. Changes in individual root Lp_r in response to pre-dawn leaf water potential ($\Psi_{predawn}$) were investigated as well. Lp_r showed a fast drop in the beginning of the water stress treatment when $\Psi_{predawn}$ was higher than -0.5 MPa. However, with $\Psi_{predawn}$ getting more negative, e.g. from -0.4 MPa to -2.0 MPa, the range of Lp_r values measured in our study maintained constant. Lp_r of well-watered plants decreased as well even though their $\Psi_{predawn}$ was maintained at a high level (< 0.1 MPa) during the period of the experiment.

In general, it is common to observe a decreased root hydraulic conductivity when plants are exposed to drought stress as demonstrated by numerous studies across various species (e.g., North and Nobel 1992 and 1998, Rieger 1995, Trifilo *et al.* 2004, Aroca *et al.* 2006, Gao *et al.* 2010). The initial decrease of hydraulic conductivity upon roots exposure to drought constraints is suggested to be a protective mechanism to prevent water from leaking back to soil with an increasing negative water potential which is lower than that of the roots (Vandeleur 2007, Aroca *et al.* 2011). As observed in our study that Lp_r tended to decline over plant developmental stages even under well-watered conditions, aging-related decrease in Lp_r has been reported by some researchers (e.g., Kramer 1983, Wells and Eissenstat 2003). Nobel *et al.* (1990) have reported an approximately linear decline of individual roots hydraulic conductivity in a desert succulent with aging from 2 weeks to 3 months.

RGM and 110R differed in the correlation between root hydraulic conductivity and root growth rate. A significant but noisy correlation between root hydraulic conductivity and root growth rate was observed in 110R, while no correlation was found in RGM. It seems that rootstock drought resistance is more related with root growth than root water uptake. But the contribution of root growth or root water uptake to plant drought resistance remains unclear.

Aquaporin gene expression

Transcript abundances of aquaporin genes in response to water stress and developmental stages were analyzed via both RT-qPCR (only *Vv*PIPs) and RNA-seq (MIP family). Comparison of gene expression data from RT-qPCR and RNA-seq revealed that there is a good correspondence in the gene expression patterns for the majority of genes between these two methods. More MIP genes were up-regulated under low level of water stress while more MIP genes were down-regulated under high level of water stress. Under well-watered conditions, significant down-regulation of certain *Vv*TIP genes were observed over development in 110R, while no significant changes in terms of MIP gene expression were observed in RGM over development.

In the case of water stress, no consistent trend has been found concerning changes in aquaporin gene expression level, and there is evidence for down-regulated, up-regulated, and unchanged expression of different aquaporin genes. For example, in maize (*Zea mays* L. cv. Potro), after 4 days without watering, the expression of *Zm*PIP1-1 gene was up-regulated, the expression of *Zm*PIP2-5 and 2-6 genes were down-regulated, and the expression of *Zm*PIP1-2, 1-5, 2-1, and 2-2 genes maintained constant (Ruiz-Lozano *et al.* 2009). In two grapevine scion varieties, the expression of *Vv*PIP2-2 gene was not modified under water stress in both varieties, while the expression of *Vv*PIP1-1 gene was up-regulated in Chardonnay but remained unchanged in Grenache (Vandeleur *et al.* 2009).

The involvement of aquaporins in root water uptake and their importance to root hydraulic conductivity have been intensively studied and demonstrated. For instance, Lovisolo *et al.* (2007) have reported a higher root hydraulic conductance associated with a higher aquaporin gene expression level in a perennial woody plant olive (*Olea europaea* L.). Antisense suppression of PIP1 aquaporin in tobacco transgenic plant resulted in reduced root hydraulic conductivity and lower resistance to water stress (Siefritz *et al.* 2002). However, our observation is not consistent with that from Lovisolo *et al*. (2007). Under low level of water stress treatment, more aquaporin genes were up-regulated in both RGM and 110R. However, Lp_r was significantly decreased even under low level of water stress. Therefore, up-regulated aquaporin gene expression did not result in any increase in Lp_r in our experiment. But is it possible that instead of facilitating water uptake, aquaporins contribute more to sustaining root growth under low level of water stress? Our analysis regarding the relationship between the expression level of *Vv*PIPs (results from RT-qPCR) and root growth rate has revealed some positive correlations between the relative expression level of several *Vv*PIP genes and root growth rate. Further analysis on the RNA-seq

results is still needed to obtain a more comprehensive understanding of the contribution of aquaporin genes to root growth under relatively low level of water stress. This analysis can also be extended to other differentially expressed genes under water stress.

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