A floral dimorphism in Nigella damascena L : genetic and molecular control, and adaptive significance

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par

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Origine génétique et moléculaire, et rôle adaptatif d'un dimorphisme floral chez *Nigella damascena* L.

Aos meus pais
(os melhores)

“Somewhere, something incredible is waiting to be known.”

— Carl Sagan, astronomer

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Personal statement

An essay on finding the meaning of fundamental research in biology

As I was preparing myself to write this thesis dissertation, I found myself trying to answer a simple question on the nature of my thesis research: what was the point of it? Luckily it seemed I needed not try too hard. Perhaps I was studying the *Nigella damascena* floral dimorphism simply for the sake of it. Indeed, three years ago that might have been a satisfying straightforward answer. I was studying the *Nigella damascena*, a plant with funny flowers and beautiful flowers. Today the answer is more complex but also more thrilling. The *Nigella* in itself has many dimensions and it may prove to be an endless well of research matter. However, in delving into my research and the concomitant bibliography I learned that no research ever stops at the model on which it is focused. Every research project in (fundamental) biology is about more than its immediate purpose, it is about the big questions. Big questions like the ever enduring mystery of the origin of life's diversity. We owe Darwin (and physics, of course) our modern integrated view of the world and life on it. And we owe it to ourselves to continue on exploring its wonder, its mysteries and its history. The *Nigella damascena*, a flowering plant which you will learn about in this dissertation, is a little wonder of life. It fits a bigger purpose.

So maybe the point was studying the mechanisms of macroevolution using the *Nigella damascena* dimorphism as a model. Or perhaps studying the evolutionary history of the flower using the Ranunculaceae species *Nigella damascena* as a model? The answer is an uncompromising "both". Explaining the diversity of living forms and how the complex features of living organisms came to be, is telling the histories of how they originated, how they became established and how they gave rise to new forms, and it requires deep understanding of their function, development and reproduction.

Fundamental research is so called because it is both elementary in its nature but also essential in its product. It is the research that asks the primary questions that fuel with deeper meaning the more applied fields of research.

Maybe you are asking yourself whether I answered any fundamental questions during my (fundamental) research, but the answer to that is not all too important. I became acquainted with one of nature's "monsters", I learned about the gene that is responsible for it and I learned of the implications of its morphology for its continuity. Studying the *Nigella damascena* funny flowers and beautiful flowers is not a stop or an end in itself. It is a step, to understanding how the multiplicity of funny and beautiful, and sometimes not so beautiful flowers of all sorts came to be. This, in turn, is a step to understanding the mechanisms involved in the multiplicity of life forms in general – the mysterious ways in which evolution created the diversity of life.

Research is a collaborative process made of small steps that brick by brick build the babel tower of knowledge. A thesis project – this thesis project – is a small step for mankind's knowledge but one giant leap for a budding scientist. Although it seems to have come to me at a late stage of this thesis, this realization of the grand scheme of things was merely the materialization of something which I instinctively knew from the very beginning of it, that I too fit a bigger purpose.

This budding scientist,

Beatriz

Introduction

"Deeper understanding confers that most precious thing – wonder."

— Brian Cox, particle physicist

Version abrégé en français de l'introduction

Les théories évolutionnistes rencontrent des limites pour expliquer l'origine de la biodiversité, dues à une compréhension incomplète des modes et mécanismes de macroévolution, soit les processus évolutifs au niveau et au-dessus de l'espèce, qui accompagnent l'origine des principales caractéristiques morphologiques qui caractérisent les niveaux taxonomiques supérieures (Gould, 1977; Gilbert *et al*., 1996; Theißen, 2009).

Bien que ne traitant pas directement d'évolution dans sa dimension historique, cette thèse s'insère dans un contexte évolutif large concernant les mécanismes potentiels de diversification de l'architecture de la fleur, et plus particulièrement de l'origine et de l'évolution des pétales et d'un périanthe bipartite, tous deux des innovations majeures. Par conséquent, l'introduction de ce travail débute par une perspective historique sur les théories évolutionnistes qui sont les plus pertinentes pour la compréhension des changements macroévolutifs. Cette perspective est suivie d'une introduction du sujet – la fleur. Des aspects morphologiques et moléculaires de la définition de la fleur sont suivis d'une perspective évolutionniste sur l'architecture florale, avec un accent particulier sur les théories de l'origine de la diversité du périanthe. Enfin, les aspects fonctionnels des structures des fleurs et du périanthe sont également discutés à la lumière des théories de l'écologie et de la biologie de la pollinisation, avant l'introduction du modèle de recherche, l'espèce *Nigella damascena* et les principaux axes de recherche explorés au cours de cette thèse.

Le contexte évolutif

Darwin a proposé que l'évolution est un processus en deux étapes, où la variation héritable aléatoire fournit la matière première sur laquelle agit la sélection naturelle, menant les organismes dans de nouveaux niveaux d'adaptation à l'environnement (Darwin, 1859). Plus tard, la théorie synthétique de l'évolution a fourni un cadre mathématique et génétique pour la description des processus à l'origine de la biodiversité, via des changements dans la fréquence des allèles provoqués par la sélection naturelle, et qui produisent des modifications subtiles du phénotype (revue dans Smocovitis, 1992, Kutschera et Niklas, 2004). Cette théorie formalise de l'idée de Darwin que les changements évolutifs se produisent

lentement par des étapes infinitésimales, dans un processus appelé gradualisme. Toutefois, cette explication de macroévolution – les processus qui mènent à la diversification des espèces et à l'origine des traits clés qui définissent les groupes taxonomiques supérieurs – par une extension au cours du temps des processus graduels de la microévolution n'as pas été consensuel, surtout à la lumière des nombreuses observations de transitions et discontinuités morphologiques et transitions abruptes entre groupes (Erwin, 2000). Des théories alternatives au gradualisme ont alors été formulées. L'une des plus importantes est le saltationnisme qui rejette l'idée de que les mécanismes de sélection naturelle agissant sur la variation graduée au niveau des populations et conduisant à un certain degré de différenciation entre les populations d'une espèce, puissent conduire ces mêmes populations plus loin vers la spéciation (Goldschmidt, 1933). En se basant sur le constat que le développement est le processus qui mène à la formation du corps et de son plan d'organisation, Goldschmidt, ainsi que d'autres promoteurs des idées saltationistes, ont reconnu le rôle des changements développementaux dans la génération de larges variations phénotypiques de la forme adulte. La forte intégration des processus du développement et les contraintes évolutives qui en résultent ont cependant conduit à la prise de conscience que la majorité des mutations développementales sont très susceptibles d'être létales, perturbant non seulement une étape dans le développement mais l'ensemble du processus. Pourtant, Goldschmidt a proposé l'hypothèse que, de temps en temps, une mutation puisse entraîner un changement significatif sans disruption complète du processus de développement, produisant ce qu'il a appelé un « monstre prometteur » (Goldschmidt, 1933). La conciliation des théories de l'évolution avec la biologie du développement a donné paissance à la biologie évolutive du développement (evo-devo), qui cherche à comprendre comment les processus par lesquels le développement traduit le génotype en phénotype affectent les potentiels et trajectoires évolutifs (Gilbert *et al*., 1996; Carroll, 2008). La prise de conscience que des changements drastiques dans les plans et les structures corporelles pourraient être obtenus par mutation d'un seul gène, et que cela se produit beaucoup plus souvent qu'on ne le pensait précédemment, est venue en renfort de l'idée saltationiste que l'évolution peut procéder rapidement et en peu d'étapes, et à la notion de Goldschmidt que tous les changements importants dans l'histoire évolutive doivent avoir une base développementale (Gould, 1977; Gilbert *et al*., 1996; Theißen, 2009).

Cependant, la faible probabilité qu'une mutation puisse créer une nouveauté sans perturber complètement le développement et que de surcroît cette nouvelle forme soit adaptative ou même seulement neutre, permettant sa survie, posent toujours un problème pour les théories saltationistes qui sont ecore loin d'être consensuelles (Theißen, 2006). Cela résulte en particulier du faible nombre d'études expérimentales ou *in natura* sur le potentiel évolutif de telles formes, et de l'absence de preuves que ces formes pourraient conduire à de nouvelles lignées évolutives (Theißen, 2006). La consolidation du saltationisme comme théorie macroévolutive dépend d'une compréhension du potentiel évolutif de ces formes, qui passe par l'étude approfondie de leurs performances écologiques et de leur comportement dans la dynamique des populations en conditions naturelles (Hintz *et al.*, 2006).

La fleur – contexte morphologique, moléculaire et développemental

Les angiospermes, avec leur histoire de diversification rapide, ont constitué pendant des années le défi le plus frappant à l'hypothèse gradualiste de Darwin, que le changement et l'innovation macroévolutive procède par des petits changements continus (Friedman, 2009). En effet, de tous les organismes vivants, les plantes à fleurs sont le groupe qui a probablement suscité le plus de controverse sur son histoire évolutive et sa diversification, notamment en raison du grand nombre d'espèces de dicotylédones qui semblent apparaître d'un coup dans le registre fossile, suggérant un événement évolutif rapide. La fleur est une innovation majeure des angiospermes et probablement celle qui a le plus contribué à la réussite de ce groupe. Dans sa forme la plus récurrente, la fleur est défini comme étant l'unité de reproduction de la plante, regroupant les organes reproducteurs femelles et mâles, respectivement les carpelles et les étamines, entouré par une série d'organes stériles appelé le périanthe (Bateman *et al*., 2006, Glover, 2007). Ce dernier présente une grande variété de formes et compositions, conduisant à une remarquable diversité globale des architectures florales. Dans le canon de l'architecture florale basé sur les eudicotylédones, il y a un périanthe bipartie avec un verticille externe composé d'organes de protection et souvent photosynthétiques (les sépales) et un verticille interne avec des organes remarquables et souvent colorées, responsables de l'attraction des

pollinisateurs (les pétales). Cependant le périanthe peut être, composé d'un seul type d'organe indifférencié appelé génériquement des tépales, ou de plusieurs types d'organes différenciés mais tous à apparence sépaloïde ou pétaloïde. La remarquable diversité d'architectures du périanthe au sein des angiospermes suggère une histoire évolutive complexe de perte et de gain d'organes, correspondant à plusieurs transitions entre les états indifférenciées et différenciées. En plus de la diversité de composition du périanthe, les pétales affichent également une remarquable diversité de forme, couleur et taille, ce qui a conduit à l'idée ancienne que ces structures ont évolué plusieurs fois indépendamment chez les angiospermes. Des analyses phylogénétiques et morphologiques semblent soutenir des origines indépendantes pour un périanthe différencié chez les monocotylédones, les Renonculacées et les eudicotylédones supérieures (Endress et Doyle, 2009; Ronse De Craene et Brockington, 2013). Basé sur un certain nombre de caractéristiques morphologiques, les pétales sont soupçonnés d'être formés à partir d'étamines ou de bractées en fonction de la lignée d'angiospermes (Kosuge, 1994; Ronse De Craene, 2007; Ronse De Craene et Brockington, 2013).

La conservation d'un plan architectural floral de base chez les eudicotylédones dérivés est au centre de la formulation du modèle ABC, un modèle de développement floral dans lequel l'action combinatoire de trois catégories fonctionnelles explique l'identité des quatre organes floraux différents : la classe A responsable de l''identité des sépales et des pétales, la classe B impliquée dans l'identité des pétales et les étamines, et la classe C dans celle des étamines et des carpelles. Il s'ensuit que, pour produire des sépales la fonction A seule suffit, alors que pour produire des pétales, l'action concertée de A et B est nécessaire. De même, pour préciser l'identité étamine, les deux fonctions B et C sont nécessaires, et pour produire des carpelles seule la fonction C est nécessaire (Coen et Meyerowitz, 1991). Des études moléculaires ont révélé la base génétique de ces classes fonctionnelles chez *Arabidopsis thaliana*. Dans cette espèce modèle, deux gènes de la fonction A ont été identifiés: *APETALA2* (*AP2*) (Kunst *et al*., 1989; Jofuku *et al*., 1994; Chen, 2004) et *APETALA1* (*AP1*) (Mandel *et al*., 1992; Gustafson-Brown *et al*., 1994). La fonction B a également été démontré être exécuté par deux gènes chez *A. thaliana*, *APETALA3* (*AP3*) et *PISTILLATA* (*PI*), deux gènes paralogues produits par un ancien événement de duplication antérieur à la divergence des angiospermes (Krizek et Meyerowitz, 1996;

Kramer *et al*., 1998). Enfin, un seul gène de la fonction C est connu chez *Arabidopsis*, *AGAMOUS* (*AG*) (Yanofsky *et al*., 1990, Bowman *et al*., 1989). Le clonage des gènes des fonctions ABC a révélé que tous codent pour des facteurs de transcription putatifs et tous sauf pour *AP2*, appartiennent à la famille de gènes MADS-box, membres de la classe de facteurs de transcription MIKC caractérisés par une séquence d'ADN hautement conservée appelée boîte MADS, qui code un motif de liaison à l'ADN (Ma et DePamphilis, 2000). Des études parallèles chez *Antirrhinum majus* ont aussi révélé l'identité des gènes de fonction B et C,, à savoir *DEFICIENS* (*DEF*) et *GLOBOSA* (*GLO*), homologues d'*AP3* et *PI*, et *PLENA* (*PLE*) homologue d'*AG* (revue dans Theißen *et al.*, 2000). Le fait que la spécification de l'identité d'organes soit réalisée par des gènes homologues de façon similaire entre les deux espèces a conduit à une hypothèse de conservation du programme ABC du développement floral au sein des angiospermes (Coen et Meyerowitz, 1991). Cependant, parce que ces deux espèces appartiennent à un groupe hautement dérivé, il faut être prudent lorsqu'on fait des inférences dans les groupes plus « basaux » d'angiospermes. En effet, ce qui avait initialement été considéré comme un degré considérable de conservation, a été par la suite contesté par plusieurs études évaluant l'applicabilité du modèle ABC à des groupes en dehors des eudicotylédones supérieures. Parmi les classes de gènes du modèle ABC les classes B et C semblent être les plus conservées, avec des homologues de ces gènes isolés chez de nombreuses espèces (Kramer et Hall, 2005; Soltis *et al*., 2006; Litt et Kramer, 2010). Cependant des observations contradictoires remettent en cause le paradigme du rôle de la fonction B dans le développement du pétale à l'échelle des angiospermes. D'une part, malgré la potentielle homoplasie des pétales dans les eudicotylédones supérieures et d'autres groupes d'angiospermes, des homologues des gènes B, *PI* et *AP3,* ont été trouvés de manière récurrente exprimés en association avec des périanthes à pétales, soutenant l'hypothèse de conservation de la fonction B au travers des angiospermes (Rasmussen *et al*., 2009; Litt et Kramer, 2010). D'autre part, des études d'expression des gènes B chez des angiospermes plus « basales » ont révélé des nouveaux patrons d'expression plus larges au sein des méristèmes floraux (Soltis *et al*., 2006), ainsi qu'une absence de corrélation entre l'expression des gène B et la présenced'organes pétaloïdes (Jaramillo et Kramer, 2004; Geuten *et al*., 2006; Landis *et al*,, 2012). Plusieurs modèles ont été formulés afin d'expliquer ces idiosyncrasies ainsi que l'évolution du programme développemental du périanthe au sein des angiospermes (détaillées

dans la version anglaise). Malgré l'importance potentielle des changements homéotiques provoqués par des altérations de gènes clés du développement dans de nombreux modèles de diversification de l'architecture florale, les mécanismes génétiques et écologiques qui permettent la mise en place de ces organismes modifiés, ainsi que leur contribution à la formation de nouvelles lignées évolutives, n'ont pas été complètement déchiffrés et constituent une étape importante dans la validation ces théories (Theißen, 2010).

Mécanismes d'évolution et diversification des traits floraux

Afin de comprendre l'évolution de la diversité florale il est indispensable de prendre en considération les aspects fonctionnels de la fleur et le rôle des ses différents traits. En effet, la fleur étant l'unité de reproduction des angiospermes, les théories actuelles semble indiquer que la diversité florale est le résultat d'un processus d'adaptation des traits floraux à différents modes de reproduction des plantes (Fenster *et al*., 2004; Kay et Sargent, 2009). En raison de leur immobilité, les plantes dépendent souvent de facteurs environnementaux biotiques et abiotiques pour se reproduire en transférant du pollen d'une plante à l'autre dans un processus appelé pollinisation croisée. Parmi les plantes à fleurs, les animaux sont les vecteurs de pollen les plus fréquents, avec lesquels elles peuvent établir des fortes relations d'interdépendance (Mitchell *et al*., 2009b). La spécialisation de ces relations entre plantes et pollinisateurs est à la base de l'hypothèse la plus acceptée pour la diversification des angiospermes (Barrett, 2010; Schiestl et Johnson, 2013). Par la promotion du transfert de pollen intra-espèce et l'isolement reproducteur (ou au niveau infraspécifique, l'homogamie), la spécialisation et les interactions plantes-pollinisateurs jouent potentiellement un rôle majeur dans la spéciation et donc dans la diversification des plantes à fleurs (Kay et Sargent, 2009). Cependant, l'idée de spécialisation des systèmes de pollinisation est en contradiction avec des observations récurrentes d'espèces visitées par un assemblage de pollinisateurs nombreux et très varié, remettant en cause le rôle des interactions plantes-pollinisateurs dans la production des conditions d'isolement reproductif nécessaires à la spéciation (Waser, 1996; Ollerton, 1996; Johnson et Steiner, 2000; Fenster *et al*., 2004). Bien que la pression sélective pour produire des organes plus attrayants ou ayant des morphologies qui favorisent

une meilleure utilisation des pollinisateurs puisse conduire à l'évolution quantitative des traits floraux tels que la taille, la forme ou le nombre (Mitchell *et al*., 2009b), les mécanismes qui conduisent à l'évolution divergente des traits floraux aux sein d'une espèce peuvent être beaucoup plus complexes (Harder et Johnson, 2009; Sapir et Armbruster, 2010). Notamment, l'importance relative des interactions plantes-pollinisateurs et la répartition géographique dans l'établissement de l'isolement reproducteuret l'évolution divergente est toujours en débat (Kay et Sargent, 2009). Ainsi, et peut-être en raison de la faible fréquence relative des cas connus de transition abrupte entre des formes florales distinctes au sein d'une espèce à l'état sauvage, la relation entre la biologie de la pollinisation et la maintenance des mutants présentant des nouveautés morphologiques qualitatives au niveau de la fleur est restée en grande partie inexplorée (Harder et Johnson, 2009).

Le dimorphisme floral chez la *Nigella damascena*

Cette thèse porte sur le dimorphisme de composition du périanthe observé chez l'espèce de Renonculacées *Nigella damascena* L. (nigelle de damas). Les plantes de *N. damascena* partagent une série de caractéristiques avec les autres membres de son genre, à savoir des feuilles très disséquées, des fleurs hermaphrodites présentant une insertion en spirale d'un grand nombre d'organes, plusieurs plans de symétrie (actinomorphie) (Endress, 1999), l'involucration des boutons floraux par des bractées, la présence de sépales colorés et de pétales de taille réduite produisant du nectar, et la production d'un fruit capsulaire (Zohary, 1983). La particularité de *N. damascena* se trouve dans la coexistence de deux variants naturels, en un dimorphisme floral de la composition du périanthe. Les fleurs du type sauvage, tel que décrit dans la définition de l'espèce, ont un périanthe différencié avec cinq sépales bleuâtres pétaloïdes et huit pétales de taille réduite et de couleur foncée et nectarifères, suivie par plusieurs séries d'étamines et cinq à six carpelles. Cette forme, classiquement appelé la forme « simple » sera ci-après dénommé le morphe [P] pour sa possession de pétales (Raman et Greyson, 1977; Zohary, 1983). La forme variante classiquement nommée «double» et appelée ici morphe [T] d'après Toxopéus (1927), produit des fleurs sans pétales mais avec un grand nombre d'organes pétaloïdes sépaliformes situés entre les sépales pétaloïdes à l'extérieur et les étamines à

l'intérieur. En plus de l'absence de pétales et de leur remplacement par des organes sépaliformes, un gradient continu de formes est observé, de sépales à sépaliformes et de sépaliformes à étamines, qui se traduit par un périanthe indifférencié et la production de formes chimériques entre le périanthe et les étamines. Ces organes intermédiaires peuvent prendre une forme d'organes sépaliformes bifides ou d'étamines membraneuses. Dans sa monographie sur la *Nigella damascena* et ses variants, Toxopéus (1927) a montré que ce dimorphisme floral est contrôlé par un seul locus, la forme [P] étant dominante et la forme [T] étant récessive. En conséquence, l'hétérozygote a le phénotype [P] (Raman et Greyson, 1977). L'existence de ce dimorphisme floral dans les populations naturelles a été signalée depuis les premières publications concernant *Nigella damascena* avec un certain nombre d'accessions de populations mixtes connues au travers de la région méditerranéenne. Cependant, la prévalence de la forme [P] et ses traits floraux partagés avec le reste des membres de son genre suggère fortement qu'il s'agit de la forme ancestrale.

Comme mentionné ci-dessus, la nigelle appartient à la famille des Renonculacées, membre de l'ordre des Ranunculales qui est le premier clade à diverger dans les eudicotylédones et groupe frère de toutes les autres eudicotylédones (APG III, 2009). La famille des Renonculacées présente une remarquable diversité de morphologie florale, consistant en une variation du nombre de pièces florales (mérisme), de l'insertion des organes (phyllotaxie) qui peut être en spiralée, verticillée ou un mélange des deux, et de la forme et composition du périanthe. Le positionnement de cette famille comme dans le clade frère des autres eudicotylédones en fait un modèle de travail privilégié pour l'étude de l'homologie des structures et des processus développementaux à l'origine de pétales et de la diversification du périanthe. En associant une transition entre périanthe bipartite et unipartite avec une perte apparente des limites entre types d'organes, le polymorphisme de périanthe de la nigelle offre une occasion unique pour déchiffrer les bases génétiques de la composition du périanthe et de la formation du pétale, ainsi que pour comprendre l'importance écologique des variations de la forme du périanthe. Cette thèse constitue un effort simultané sur ces deux questions. Ainsi, le premier objectif de ce projet de thèse était d'identifier l'origine moléculaire du dimorphisme floral en utilisant une approche de type gène candidat, incluant une étude comparée approfondie des profils d'expression génique chez les

deux formes et une validation fonctionnelle des gènes exprimés de manière différentielle. La compréhension des mécanismes moléculaires contrôlant le développement et la différenciation du périanthe est très importante pour comprendre l'origine de la diversité génétique et l'évolution de la forme du périanthe. Identifier les gènes clés responsables de la présence / absence de pétales représente aussi une étape importante dans le décryptage du réseau génétique contrôlant la formation des pétales chez les eudicotylédones basales. Le deuxième volet de cette thèse concerne la signification évolutive du dimorphisme. Notamment, étant donné que les différences morphologiques observées entre les deux formes affectent des traits potentiellement impliqués dans l'attraction des pollinisateurs, comme la présence de pétales nectarifères et le nombre d'organes potentiellement attractifs, ce polymorphisme est susceptible d'affecter le comportement des pollinisateurs et la capacité de reproduction de la plante. En conséquence, dans la deuxième partie de ce projet, nous avons étudié l'impact du dimorphisme sur les interactions plantes-pollinisateurs, et ses conséquences sur le succès reproducteur.

The evolutionary context

Wonderful mysteries

Despite the great advances made in the evolutionary biology field since the publication of Darwin's seminal work, life on earth has kept many elusive mysteries. The most wonderful of these enduring challenges is perhaps the origin of complex forms and the evolution of their great diversity (Lenski *et al.*, 2003). As Theißen (2006) amusingly put it, "Why did bacteria not just give rise to more and more optimized and better and better adapted bacteria forever, but to mushrooms, monkey-flowers and man?"

The fundamental challenge of evolutionary theories in explaining the origin of such biodiversity can be pinpointed to our still poor understanding of the modes and mechanisms of macroevolution. Although originally pertaining to the evolutionary processes that lead to the origin of species and higher taxa, macroevolution is now most commonly used to refer to the evolutionary processes at and above the species level, that accompany the origin of the key morphological features that characterize higher taxonomic levels (Gould, 1977; Gilbert *et al.*, 1996; Theißen, 2009).

While not dealing directly with evolutionary histories, this thesis inserts itself in a deep evolutionary context regarding the potential mechanisms of flower architecture diversification, particularly in what concerns the origin and evolution of petals and of a bipartite perianth, both of which can be categorized as major innovations of the flower structure. Therefore in this introduction I chose to present a historical perspective of the aspects of evolutionary theories that are most relevant to the understanding of macroevolutionary change. This perspective is followed by an introduction of the subject matter – the flower. Morphological and molecular aspects of flower definition will lead way to an evolutionary perspective on flower architecture, with a special accent on the theories on the origin of perianth diversity. Finally the functional aspects of flower and perianth structures will also be discussed in light of ecology and pollination biology theories, before introducing the research model *Nigella damascena* and the main lines of research explored during this thesis.

Darwin's legacy

Perhaps the most striking among Darwin's suggestions that stood the test of time, is the idea that evolution is a two step process, where heritable random variation provides the raw material on which natural selection acts, leading organisms into new stages of adaptation to environment (Darwin, 1859). That innovating thesis gained extraordinary support some 80 years later, from the combination of population genetics theories with observations on the fields of embryology, systematics, biogeography and paleontology that gave rise to the **modern evolutionary synthesis** (reviewed in Smocovitis, 1992). This modern perspective on evolution drew heavily from the statistical and mathematical population genetics theories to explain evolution as the result of changes in allele frequency forced by natural selection, which produce subtle modifications of phenotype. It also provided a concrete genetic basis for the previously obscure heritable substrate of evolution by integrating the concepts of gene and mutation as a source of variation (Dobzhansky, 1963a). But more importantly, this synthetic theory reiterated Darwin's idea that evolutionary change occurs slowly but steadily, by infinitesimal steps of adaptation, in a process called **gradualism** (**Box 2**) (Kutschera & Niklas, 2004).

Over the following years biologists have had no major issue with the modern synthesis, especially because its strong mathematical foundation provided a solid basis for the description of the **microevolutionary** processes that act at the population level. That is, the origin of diversity below the species levels – subspecies and varieties – by adaptation to local environment. However, this theory wasn't without fault and perhaps its greatest liability lays in its attempt to explain **macroevolution** as an extension of microevolution through time. In other words, it used the same gradual processes of organismic adaptation via small shifts in gene frequencies, to explain the diversification among species and the origin of the key innovatory traits that define the upper taxonomical groups. Although some transitions between different groups may in fact be smooth, suggesting an accumulation of microevolutionary processes in an infinite continuum of graded steps, a myriad of abrupt transitions and discontinuities can also be seen across all levels of hierarchical organization, strongly conflicting with the accumulation of microevolution hypothesis (Erwin, 2000). In light of such discrepancies in the rates and processes of diversification, the key to understanding the concept of macroevolution may

be connected to its association with the origin of the **key innovations**, that is, the novel structural features and body plan organizations that define major groups of living organisms (see **Box 1**). The frequent observations in the phylogenetic history of abrupt branching patterns between higher taxa (Vergara-silva, 2003), and the lack of compelling evidence in the fossil record for a gradual evolution of key innovative features and body plans (Gould, 1977), have recurrently relaunched the debate on the mechanisms that bring about such novelties, i.e. the mechanisms of macroevolution.

Today, it is widely accepted among evolutionary biologists that macroevolution is not simply microevolution extrapolated and that major structural transitions can occur rapidly without a continuous series of gradual steps.

Box 1. The "key innovation" or "novelty" concept

Despite giving a framework definition of novelty as a feature of the phenotype that cannot be homologized to any precursor structure, Theiβen (2009) also highlighted the difficulty in defining such a concept, as ancestral phenotypic states may not always be ascertained. Wilson (2011) made a good case for the definition of key innovations in stressing not what they are made of, but their consequences, namely their role in the generation of diversity. Wilson highlighted the capacity of some novelties to increase the evolutionary potential of the species in which they appear and to lead those lineages possessing them into greater diversity. Visually put, after the appearance of one such key innovations, a clade containing it tends to be more species rich than its sister clades without it. Today, in a more simplistic manner, key innovations or novelties can be defined as strong phenotypic transitions that accompany the macroevolutionary processes that produce the chief features used in defining major taxonomic clades, such as new body plans and body

"Evolutionary concepts evolve" (Dobzhansky, 1963b)

The failure of the modern synthesis and other neo-Darwinian gradualist theories in explaining the origin of complex body features and evolution of body plan diversity, provided the opportunity for the formulation of alternative theories for the mechanisms of macroevolution. Perhaps by virtue of its initial controversy, the most prominent of these is the **saltationism** theory (**Box 2**) defended by Richard Goldschmidt (1933). Although agreeing that mutation rates may be high enough to provide sufficient variation for natural selection to act upon producing a certain degree of differentiation among populations of a species, Goldschmidt rejected the hypothesis that this same gradual

mechanism could lead populations a step further into speciation. He argued that the changes required for the differentiation of new species are far greater than those obtained by the gradual mechanisms of subspecies and population differentiation (Goldschmidt, 1933). At the time this view generated great controversy among the population geneticists who strongly defended the gradual genetic basis of evolution, i.e. the notion that all processes of evolution can be mathematically explained by the microevolutionary processes of gene frequency changes that originate varieties within populations (Gilbert *et al.*, 1996). Nevertheless, Goldschmidt was not alone in his view, with many scientists before and after him sharing some of his beliefs on discontinuous evolution (reviewed in Levit *et al.*, 2008). Darwin's contemporary Thomas Huxley had previously pointed out the frailty in an exclusively gradualist mechanism of evolution, and the independence between the concepts of natural selection and gradualism. Hugo deVries, one of the early geneticists, had also proposed a form of saltationism after the rediscovery of Mendel's laws. His theory, named mutationism, postulated that new species can only arise by sudden non-adaptive variation without gradual transitions. And the geneticist William Bateson (1894) wrote an extensive treaty on the discontinuous nature of variation and species, in which he noticed the dramatic homeotic nature (defined below) of some morphological changes.

Box 2. Some evolution theory definitions

Gradualism – the prevailing view in Darwin's and neo-Darwinian theories that evolutionary processes both at the species level and above, even those that produce strong phenotypic innovations, are the product of an almost infinite number of steps between gradually transitioning phenotypical states. Accumulated over time these infinitesimal steps eventually generate the unique and complex structures that define the higher taxa.

Saltationism – opposing view that profound evolutionary novelties result of sudden discontinuous change and true species are separated by 'bridge-less gaps' arising abruptly by macromutation (Goldschmidt, 1933).

Macromutation – mutations in genes controlling developmental pathways that result in changes in developmental processes that have large effects in the adult phenotype.

Pre-adaptation – a working hypothesis to explain the discontinuous nature of evolutionary novelty via a theoretical transitional form which has a different function and adaptive value from the final structure (Gould, 1977). Goldschmidt elaborated on the value of such pre-adaptive states for the colonization of new environments by a species (Goldschmidt, 1933).

The originality of Goldschmidt's assertions came from his early recognition of the role of developmental changes in the generation of strong phenotypic variations in the adult form – which he called **macromutation** (**Box 2**), a concept later reinforced by the new synthetic theories of evolutionary development (evo-devo, see below). Goldschmidt acknowledged some of the early embryologists pioneering ideas on the strong integration and evolutionary constraints of development as the process that brings about body plan organization. This view came to prominence later on in the work of Gould and Lewontin (1979) who wrote a critique of the adaptationist views of evolution and the importance placed on selection on individual traits as the driving force of species differentiation, and emphasized the role of integrated relationships within the segments and parts of the organisms body plans and developmental processes in determining evolutionary pathways. Acceptation of the limitations imposed on development led to the realization that the majority of developmental mutations, no matter how small, are very likely to be lethal, disrupting not just a single step in the development but the whole process. Yet, Goldschmidt did not discard the hypothesis that every once in a while a single mutation in an independent process of development could result in developmental change without its total disruption, producing what he called a 'hopeful monster'. He argued that the successful establishment of such new forms could lead to the evolution of diverging lineages, illustrating how it is possible to bridge the gap between very distinct taxonomic groups with one step (Goldschmidt, 1933).

Goldschmidt's vision, and particularly his awareness of the potential of relatively small developmental change in the production of great adult phenotypic variability, was only acknowledged some fifty years later. First in the work of Gould and Eldredge (1972), and Bateman and DiMichelle (2002), who collectively proclaimed the *return of the 'hopeful monster'*, and later with the advent of evolutionary developmental biology (evo-devo) (reviewed in Theißen, 2009). This synthetic discipline aimed at reconciling the evolutionary theory with insights from the developmental biology field, in order to understand how the modes by which development translates genotypes into phenotypes affect evolutionary potentials and trajectories (see **Box 3**) (Gilbert *et al.*, 1996; Carroll, 2008). One of its chief pillars was the rediscovery of homeotic mutants, forms in which one body segment is correctly

formed but wrongly placed, along with the discovery of the *Drosophila* homeobox genes, a class of regulating genes that discretely control the patterning of body plans and structures through developmental processes (True, 2005). The realization that drastic changes in body plans and structures could be obtained by mutation of single genes, and that this occurs much more frequently than previously thought, lent great support to the saltationist idea that evolution can proceed in single steps, and to Goldschmidt's notion that all important changes in evolutionary history must have a developmental base (Gould, 1977; Gilbert *et al.*, 1996; Theißen, 2009).

Box 3. The evo-devo revolution

The classical neo-Darwinian view of evolution as the result of changes in gene frequency brought about by natural selection (along with mutation, drift and migration), provided a strong working basis for the study and understanding of the processes that lead populations and species into new adaptive states. However, it left a major gap when it came to providing an explanatory scenario for the origin of evolutionary novelties, i.e. remarkable new body structures and new states of body plan organization. The evo-devo rationale aimed at bridging this gap. The evo-devo theory is strongly based on the principle that adult multicellular organisms arise from a single cell through the intricate process of development. Therefore, morphological changes in adult forms must be the product of developmental changes. Given that developmental processes are largely under genetic control, morphological novelties must arise by mutation or changes in the regulation of those key genes controlling development. Thus, the study of evolutionary change and morphological diversity is not so much based on the evolution of gene frequencies at the population level but on the phylogenetic history of those genes.

Hopeful homeotic monsters

In spite of the attention it garnered, Goldschmidt's theory of 'hopeful monsters' still posed a great challenge for saltationists, particularly when it came to explaining the mechanisms for their success. Goldschmidt himself (Goldschmidt, 1933) realized that the odds were against the apparition of such a mutation (the probability of a mutation not being completely disruptive being low) and subsequent survival of such forms (the likelihood of it being adaptively beneficial or even neutral being even lower). However, he argued that there could be rare circumstances in which these 'monsters' could withstand natural selection and persist in a population long enough to establish themselves in a new

environment. Drawing from the concept of **pre-adaptation** (**Box 2**), Goldschmidt argued that the 'hopeful monster' would be particularly fit not for the environment in which it appeared, but for a different and yet unexplored niche. Once there, it could establish itself and continue to evolve, further adapting to the new environment through mutation and selection (Goldschmidt, 1933; Gould, 1977). This idea was reviewed and deepened by Bateman and DiMichele (2002) who developed the concept of prospecies, equivalent to the 'hopeful monster', as the product of profound phenotypic change over one generation, and the starting point of a potentially independent evolutionary lineage. Their hypothesis was that, because the fitness of prospecies is in most cases too low, their establishment is most likely to occur under temporary release from selection in low competition scenarios. After an initial period under such circumstances, the new form would achieve a reasonable frequency and could re-enter the competition, continuing to improve through mutation and selection in a neo-Darwinian fashion.

Although promising, these theories, as well as the more general theories of saltational evolution, have yet to achieve general acceptance as not enough studies on the real evolutionary potential of natural discontinuous forms, and no real evidence that such forms could establish themselves in the wild giving rise to new evolutionary lineages have been successfully obtained (Theißen, 2006). Perhaps in an ironic twist, evo-devo theories on their own provide little insight into the potential of homeotic mutants/monsters in natural environments, a condition upon which depends the general acceptance of a saltational mode of evolution. Instead, the hopeful fate of homeotic mutants/monsters lies in the study of the ecological aspects and the population dynamics mechanisms of their performance in natural conditions (Hintz *et al.*, 2006).

The flower –morphological, molecular and developmental context

Why flowers?

Of all the living organisms, flowering plants compose the group that has likely generated the most controversy over its evolutionary history and diversification. Darwin's own enthrallment with the subject is notorious and has been a staple for flower evolution bibliography for over 150 years. The apparently abrupt radiation of angiosperms, evident in the overwhelming number of dicotyledons species which appears unprecedented in the fossil record, was to Darwin a "most perplexing phenomenon". But despite the deep value of angiosperm diversity as research matter for evolutionary biologists, the real and seminal reason why Darwin called it the most "extraordinary" aspect of plant evolution is not its colorful history, but the fact that angiosperms with their history of rapid diversification constitute the most striking challenge to his gradualist assumption that macroevolutionary change and biological innovation proceed by small and continuous changes (Friedman, 2009).

What's in a name?

The flower is the landmark feature of angiosperms and, in good key innovation custom (see **Box 1**), it is the trait that likely most contributed to the evolutionary success of this group. Despite their ubiquity, the very definition of flower still remains a challenging task, particularly because it embraces a remarkable diversity of shape, composition and organ types. In its more recurrent form, the flower is defined as the unit of reproduction of a plant, regrouping the female and male reproductive organs, respectively the carpels and stamens, surrounded by a series of sterile organs called the perianth (Bateman *et al.*, 2006; Glover, 2007). If the organization of the reproductive structures is relatively conserved – carpels at the center and stamens encircling them – which is likely due to a strong functional constraint on their evolutionary potential, the perianth – a sterile and functionally labile structure – exhibits a wide variety of shapes and compositions leading to an overall remarkable diversity of floral architectures across angiosperms.

The canon of flower architecture, based on the most represented group of extant angiosperms, the core eudicots, has a **bipartite** perianth with an outer or first whorl (calyx) of plain and often green organs, called the **sepals**, and an inner or second whorl (corolla) of conspicuous and often colored organs, called the **petals**. The general morphological characteristics of perianth organs have been classically associated with organ function: the photosynthetic sepals providing protection for the developing floral buds, and the showy petals being responsible for the attraction of pollinators.

Despite the recognition of a general pattern, perianth forms across angiosperms are marked by an incredible diversity at the architecture, composition, shape and color levels. The most common alternative to a bipartite perianth, for example, is a **unipartite** one, composed of a single undifferentiated organ type. These organs, despite being generically called **tepals**, neither sepals nor petals, are commonly appended with the designations **petaloid** or **sepaloid** according to their general aspect, showy or plain. In addition to unipartite perianths with a single organ type, it is also possible to have a perianth where all organs are brightly colored and showy but can be distinguished into outer and inner organs based on their morphology as in a bipartite perianth. In these cases the outer organs may be called petaloid sepals to indicate the presence of showy organs in the first whorl in addition to the inner petals. Hence, while the term petal is most closely associated with the notion of a differentiated perianth, the term **petaloidy** does not refer to the presence of petals, but rather to a visually striking morphological state of floral organs which can occur outside the second floral whorl and is usually associated with the production of colored pigments and papillate epidermal cells (Ronse De Craene & Brockington, 2013).

The molecular side of the flower

Despite the great morphological diversity of flowers across angiosperms, the conservation of a basic floral architectural plan in higher eudicots is at the center of the formulation of the ABC model of floral development and organ identity. This model for floral organ identity specification was developed in parallel in the core eudicot model species *Arabidopsis thaliana* and *Antirrhinum majus*, upon observation of a curious group of homeotic mutants exhibiting alternative floral architectures.

Three classes of mutants could be discerned among them, A, B and C. Class A mutants have carpels in the place of sepals, and stamens in the place of petals, class B mutants have sepals in both perianth whorls and carpels in both inner whorls, and class C mutants have sepals and petals at the place of stamens and carpels respectively (Coen & Meyerowitz, 1991; Bowman *et al.*, 1991). Additionally, class C mutants also show a disruption of floral meristem determinacy resulting in an increase in the number of floral pieces, a phenomenon commonly called 'double flower' (Dubois *et al.*, 2010). The careful interpretation of these mutants led to the formulation of a simple developmental model, in which the combinatorial action of three functional classes explains the specification of the four different floral organs (**Figure 1**). If in class A mutants normal perianth specification is disrupted, then A function must be related to the identity of sepals and petals. Similarly, if class B mutants fail to produce petals and stamens, then the B function pertains to the identity of petals and stamens. Finally, class C mutants suggest a function of this gene class in stamen and carpel identity, as well as meristem determinacy. It then follows that in order to produce sepals the A function alone suffices, whereas to produce petals the concerted action of A and B function is required. Likewise, to specify stamen identity both B and C functions are needed, and to specify carpels the C function alone is required (Coen & Meyerowitz, 1991).

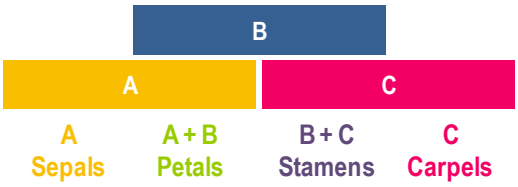

Figure 1. Simplified schematization of the ABC developmental model of floral organ identity specification. Based on Coen & Meyerowitz, 1991.

Molecular analysis studies have revealed the genetic basis for these functional classes in *Arabidopsis thaliana*. In this model species, two A function genes have been identified: *APETALA2* (*AP2*) and *APETALA1* (*AP1*). The *AP2* gene is initially expressed in all four whorls of developing flowers but is later believed to be repressed in whorls 3 and 4, becoming restricted to the first and second whorls were it is required to specify sepal and petal identities respectively (Kunst *et al.*, 1989; Jofuku *et al.*, 1994; Chen, 2004). In addition to its role in sepal and petal development, the *AP1* gene has an

additional and crucial role in the specification of floral meristem identity. Accordingly, *AP1* is initially expressed at an early stage of floral development in a broad fashion across the meristem and only later restricted to whorls 1 and 2 (Mandel *et al.*, 1992; Gustafson-Brown *et al.*, 1994). The B function has also been shown to be performed by two genes in *A. thaliana*, the *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), two related genes issued from an ancient gene duplication event predating the angiosperm lineage (Krizek & Meyerowitz, 1996; Kramer *et al.*, 1998). *AP3* and *PI* are both expressed in the petal and stamen primordia and their products form obligate heterodimers in order to regulate expression of downstream genes implicated in petal and stamen development, as well as each other's (Goto & Meyerowitz, 1994). Finally, only one C function gene is known in *Arabidopsis*, the *AGAMOUS* (*AG*) gene (Yanofsky *et al.*, 1990). Similarly to the *AP1* gene, *AG* has a double function specifying not only stamen and carpel identity but also floral meristem determinacy (Bowman *et al.*, 1989). Parallel studies of ABC functions in *Antirrhinum* revealed the identity of both B and C function genes. *Antirrhinum* B function is performed by *AP3* and *PI* homologs *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) respectively, while C function is performed by *PLENA* (*PLE*), an homolog of *AG* (reviewed in Theißen *et al.*, 2000). The fact that organ identity specification is performed by homologous genes in a similar fashion between the two distinct species, led to the initial assumption of broad conservation of the ABC program of floral development across flowering species (Coen & Meyerowitz, 1991).

Cloning of these ABC function genes revealed that all encode putative transcription factors and, except for *AP2*, all belong to the MADS-box family of genes (Ma & DePamphilis, 2000). The members of this family share a highly conserved DNA sequence, called the MADS-box, which encodes a DNA-binding motif. The MADS-box genes involved in floral development belong to a special class of transcription factors called MIKC-type proteins for the presence of four different domains: the MADS (M), intervening (I), keratin-like (K) and C-terminal (C) domains. The MADS-domain, the most conserved region, is required for DNA-binding and protein dimerization. The I-domain is also required for DNA binding of dimer forming proteins and it is believed to influence the specificity of the DNA-binding dimer formation. The K-domain is involved in the mediation of interaction between MIKC-type proteins. The C-terminal domain, the least conserved region, acts as a

stabilizing/enhancing factor in K-domain mediated protein interactions (MIKC domain functions were reviewd in Kaufmann *et al.*, 2005).

In more recent years the ABC model has been extended to two new classes of genes, the D and E functions. The D function genes also encode MADS-box proteins and are required for ovule development being believed to specify ovule identity in cooperation with the C function (Angenent *et al.*, 1995). The E function class of genes has revealed a group of three MADS-box transcription factors that are not only required for the B and C functions, but also allow us to understand how the combinatorial action of the ABC and E genes can ectopically specify floral organ identity (Honma & Goto, 2001). This was particularly relevant because while the combinatorial action of the ABC functions had previously been shown to be necessary it was not sufficient to specify floral organ identity. The addition of the E function finally permitted to understand the specification of floral organs from a vegetative part such as the leaves. The E function genes, *SEPALLATA1* (*SEP1*), *SEP2* and *SEP3*, have been included in a more recent revision of the floral model of organ identity – the ABCE model. Studies of protein interaction among the ABCE proteins finally led to the proposal of a "floral quartet" model directly linking floral organ identity to the action of four different tetrameric transcription factor complexes (Theißen & Saedler, 2001; Smaczniak *et al.*, 2012).

In addition to the genes responsible for floral organ identity, other genes and mechanisms implicated in the regulation of floral meristem spatial organization have been discovered. Central to the formation of discrete whorls of organ identity in the flower, is the antagonism between A and C functions which maintains the boundary between the second and third whorls via mutual repression of those gene classes. Additional genes recently discovered to participate in the formation and maintenance of boundaries between organ whorls are *SUPERMAN* (*SUP*) and *RABBIT EARS* (*RBE*). *SUP*, which encodes a putative transcription factor, is required for the maintenance of the boundary between the third and fourth whorls (stamens and carpels) via a cell proliferation regulation mechanism (Sakai *et al.*, 1995; Yun *et al.*, 2002). *SUP* has also been shown to have direct and indirect roles on the expression of B function genes *AP3* and *PI* (Yun *et al.*, 2002). *RBE* encodes a SUP-like protein involved in the early development of second whorl organs by regulating the boundaries between these

organ primordia as well as the boundary between the second and third whorls via restriction of *AG* expression to the inner whorls (Takeda *et al.*, 2003; Krizek *et al.*, 2006).

While the region specific expression of floral homeotic genes is established through negative interactions, their initial activation is mostly dependent on positive regulation mechanisms. In *Arabidopsis*, the transition from vegetative to reproductive growth is the result of the integration of a series of environmental, physiological and developmental pathways, whose signals in turn converge into the activity of a few master regulatory genes of floral development such as the above mentioned *AP1* and the gene *LEAFY* (*LFY*) (Liu & Mara, 2010). These two genes act together within the inflorescence to promote floral meristem identity of lateral meristems (Weigel *et al.*, 1992; Bowman *et al.*, 1993). *LFY* encodes a plant specific transcription regulator expressed very early in the floral primordium along with several other genes with whom it partners to activate in a temporal and region specific manner the ABCE homeotic genes (Liu & Mara, 2010). *AP1* is one of the partners of *LFY* and together they have been shown to activate the expression of *AP3*, *PI* and *AG* (Weigel & Meyerowitz, 1993). Other co-factors of *LFY* are *SEP3*, required for the temporal control of *AP3*, *AP1* and *AG* activation; *UNUSUAL FLORAL ORGANS* (*UFO*), an F-box protein encoding gene which is expressed transiently in a similar domain to *AP3* and *PI* and is necessary for their activation; *WUSCHEL* (*WUS*) gene, a homeodomain protein responsible for the maintenance of the stem cell pool in apical shoot and floral meristems, that is required as co-factor of *LFY* for the activation of *AG* in the inner whorls of the flower primordium; and *PERIANTHA* (*PAN*), a bZIP protein encoding gene also involved in the direct activation of *AG* (reviewed in Liu and Mara, 2010).

Conservation of developmental programs

The ABCE model provides a solid working base for the study of floral developmental genetics, with many powerful predictions and testable hypotheses. However, because it has been designed based on observations made in highly derived species, one has to be cautious when making inferences in lower groups of angiosperms. Consequently, what had initially been viewed as a considerable degree of conservation, has been subsequently challenged in recurrent studies testing the applicability of the

ABC model to groups outside the core eudicots. Among the functional gene classes of the ABCE model the B, C and E classes seem to be the most conserved with homologs of these genes being isolated from a range of species, while A class homologs with functional roles on flower development are yet to be found (Kramer & Hall, 2005; Soltis *et al.*, 2006; Litt & Kramer, 2010). Therefore, developmental molecular clues into the evolution of petals and perianth differentiation must be based on the study of B function conservation.

Contrary observations punctuate the paradigm of B function in petal development across angiosperms. In model core eudicots *AP3* and *PI* are expressed in a specific manner in the second whorl of the perianth (as well as in the third whorl), where their activity is continuously required for petal specification identity and the proper development of mature petal morphological features. In addition, the heterotopic expression of B genes in the first whorl is capable of inducing ectopic petal formation (Krizek & Meyerowitz, 1996). Despite the likely homoplasy of petals in core eudicots and other outside groups, homologs of the *AP3* and *PI* B genes have been recurrently shown to be expressed in association with petalous perianths across angiosperms, apparently supporting a conservation of B function (Rasmussen *et al.*, 2009; Litt & Kramer, 2010).

On the other hand, studies of B gene expression in basal angiosperms have revealed a previously unknown complexity of patterns and dynamics. Most notably, unlike in the core eudicots, activity of *AP3* and *PI* homologs in basal angiosperms is not restricted to certain whorls but is found in broad domains across floral meristems and their continued expression in later stages of primordia differentiation is not required for the proper petal development (Soltis *et al.*, 2006). Additionally, although B gene expression can be found in first whorl petaloid organs in some species (Bowman, 1997; Kramer *et al.*, 2003), other studies have shown a lack of association of B gene expression with the occurrence of petaloid organs outside the second whorl, or an expression of B genes in non-petaloid second whorl organs (Jaramillo & Kramer, 2004; Geuten *et al.*, 2006; Landis *et al.*, 2012). These observations have lead authors to question the role of B genes in specifying petals and petaloidy, and to advance an hypothesis on the decoupling of petaloidy and B gene expression in less derived angiosperms (Ronse De Craene, 2007). The idea that B genes expression does not imply the

production of petals or petaloidy was elegantly incorporated in the '**regional specification'** model (Irish, 2009). This model reiterates the idea that specification of distinct inner perianth organs in association with the expression of B gene orthologs in that domain does not necessarily lead to the production of petals. Therefore, the ancestral role of B gene homologs may not be the specification of organ identity but of a region within the perianth (**Figure 2**, *p.* 20) (Drea *et al.*, 2007; Irish, 2009).

Molecular developmental theories of perianth architecture evolution and diversity

The remarkable diversity of perianth architectures across angiosperms has long suggested a complex evolutionary history of organ loss and gain, eliciting several transitions between the undifferentiated and differentiated states. In addition to the diversity of perianth compositions, petals also display a remarkable diversity of form, color and size across angiosperms, which have led to a long standing belief that these structures have evolved several times across angiosperm lineages. Phylogenetic and morphological analyses both seem to support independent origins for the differentiated perianth in the monocots, the Ranunculaceae and in core eudicots (Endress & Doyle, 2009; Ronse De Craene & Brockington, 2013). Bowman (1997) pointed out that evolution of a bipartite perianth from an undifferentiated state, could occur by differentiation of the existing perianth into two distinct organ types, or alternatively, by conversion of stamens to petaloid organs. Based on a number of complex morphological features such as development rate, epidermal cell identity and venation pattern, petals are believed to have arisen as modifications of stamen-like structures in a process of andropetaloidy, or to be derived from bract- or sepal-like organs, bracteopetaloidy, depending on the angiosperm lineage (Kosuge, 1994; Ronse De Craene, 2007; Ronse De Craene & Brockington, 2013).

Box 4. Flower origin

Several difficulties underline the definition of the ancestral character states of the angiosperm flower: lack of continuous morphological data between extant groups and fossil record; unresolved relationships among angiosperm groups, particularly basal angiosperms, unresolved relationships among the sister groups to angiosperms; a large morphological gap between gymnosperms and angiosperms (Frohlich, 2006; Soltis *et al.*, 2008). Despite considerable disparity between molecular studies and fossil record analysis in the calculation of the time of origin for angiosperms, the most compelling evidence places their divergence from the sister group gymnosperms somewhere between 130 and 300 Mya (Frohlich, 2006; Soltis *et al.*, 2008; Bell *et al.*, 2010). Among the extant angiosperms the monotypic *Amborella* is placed as the sister group to all other angiosperms, followed by the Nymphaeales and Austrobaileyales (Qiu *et al.*, 1999; Glover, 2007; Soltis *et al.*, 2008). Several theories for the **origin of the flower** have been proposed based on compared morphological and molecular studies of extant gymnosperms and those basal angiosperms groups.

The **"Mostly Male" theory**, based on the retention of a single copy of the *LEAFY* (*LFY*) gene in angiosperms, hypothesizes that the bisexual flower organization derives from the male rather than female structures of ancestral gymnosperms (Frohlich, 2003). An ancient duplication of *LFY* occurred before the divergence of major gymnosperm lineages producing the *Needle* and *Leaf* copies that are expressed in female and male cones respectively, yet only one copy of *LFY* can be found in angiosperms. Retention of the *Leaf* copy in angiosperms led to a model of the ancestral flower in which male specification function was primarily retained and only a small set of genes responsible for female identity was kept for ovule development.

B genes have been isolated in gymnosperms and have been shown to be expressed exclusively in the male reproductive structures of this group of plants. Along with evidence for their ability to complement stamen identity in angiosperms, this suggests an ancestral function for B genes in a switch-like sex determination mechanism. When B genes are expressed they specify male cones and when absent female cones are formed (reviewed in Theißen & Becker, 2004). These observations led Theißen and Becker (2004) to propose the **"out of male" and "out of female" hypothesis** for origin of bisexual flowers. A hermaphroditic flower precursor could appear either through upregulation of the male specifying B-genes in female cones of ancestral seed plants or reciprocally downregulation in the male cones.

Baum and Hileman (Baum & Hileman, 2006) also propose a scenario for the origin of flowers accounting for the origin of bisexuality based on homeotic mechanisms. Baum and Hileman pointed out that in modern flowers, male structure specification requires the activity of both B and C function whereas the identity of female structures requires C function alone. In simple terms, if B and C function genes were expressed in male cones, then the authors propose that a local increase of C function activity in the terminal region could competitively eliminate the B function and specify a female region within a male cone producing the flower bisexual precursor.

Reconstructions of ancestral character states by parsimony analysis on molecular phylogenies have constituted a means for the reconstruction of the ancestral flower and its early evolution, providing a frame for several working theories for the evolution of the perianth (see **Box 4** for theories on the flower origin). Depending on taxon sampling however, these studies can yield different results making this an ever changing work in progress. Whether the origin of the perianth accompanied the origin of the flower or not, it is largely accepted that at some point in early flower evolution the perianth appeared as a set of sterile structures loosely integrated in the flower architecture (Endress, 1994; Endress & Doyle, 2009). The consensual view is that this early perianth was indeed composed of undifferentiated, entirely sepaloid organs, organized in a variable phyllotaxis of several whorls or several spiraled series (Endress & Doyle, 2009).

The ancestral undifferentiated perianth hypothesis concurs well with the observed lack of distinct whorls of organs in basal angiosperm flowers. In these early diverging groups, floral organs are often inserted in a spiral of intergradating forms: from bracts to outer tepals, to inner tepals, to stamens and to carpels (Buzgo *et al.*, 2004). This gradual transition of organ identities is commonly accompanied by broad expression patterns of the organ identity genes homologs, particularly in the B function (Kim *et al.*, 2005). These observations led to the proposal of a new model for the evolution of the flower developmental program called the **"fading borders" model** (Buzgo *et al.*, 2005). According to this model, the gradual intergradation of floral organ identities in basal angiosperms is the result of gradients in the expression levels of organ identity genes and represents the ancestral condition of the angiosperm flower, whereas the discrete and unambiguous organ identities of the core eudicots bipartite perianth represent a derived condition (**Figure 2**) (Soltis *et al.*, 2007). The broad expression domains of organ identity genes in basal angiosperms also represent a more labile and plastic state of the flower developmental program which could explain the great diversity of floral forms outside the core eudicots (Soltis *et al.*, 2007). The confinement of organ identities in floral whorls in higher angiosperms is believed to be the result of a process where the broad expression domains and flexible functions of homeotic genes seen in the fading borders model were canalized into the discrete domains and precise organ identity functions seen in the eudicot ABCE model (Chanderbali *et al.*, 2010).

Theiβen and Melzer (2007), for example, invoked a mechanism of positive auto-regulation, via the evolution of regulatory sequences in the promoter regions of homeotic genes to explain the origin of sharp boundaries between whorls. In such circumstances positive auto-regulation is expected to lead to the amplification of small differences in expression levels over the gradients of gene activity creating discrete domains of expression. Alternatively, the restriction of functional activity to sharp domains could result from the evolution of obligatory protein-protein interactions, such as the obligate heterodimerization of B genes *AP3* and *PI* (Winter *et al.*, 2002). In either case the final result is the formation of a discrete domain of action of the B genes within the perianth that could pave the way to the evolution of a new identity program and the origin of a differentiated perianth.

The establishment of a strict four-whorled floral structure could be expected to lead to a certain level of evolutionary constraint and result in a higher degree of conservation. However, despite sharing this conserved basic plan, higher angiosperms still display an overwhelming diversity which is visible both in changes at the level of flower architecture, for example new plans of symmetry, or as secondary elaborations, such as change in color, size or shape of organs. One explanation for this diversity could stem from the fact that this evolutionary step also coincides with major duplications in almost all lineages of MADS-box organ identity genes (Litt & Kramer, 2010). In many lineages gene duplication was followed by modifications to expression patterns and/or function in new paralogs, which could provide a genetic basis for novelties and secondary alterations to the flower structure. Theißen & Melzer hypothesis proposed that new sources of variation to the floral structure can evolve via the establishment of new interactions between the ABC genes and their targets or with new co-factors (Theißen & Melzer, 2007). But perhaps the most powerful mechanism when explaining the diversification of flower architecture and organ identities is one that requires only that simple changes occur in the expression domains of the floral homeotic genes. Such a mechanism has been proposed by different authors under the name of **"sliding boundaries"** (Bowman, 1997; Kramer *et al.*, 2003). This model has acquired particular meaning in relation to the transitions between differentiated and undifferentiated perianth, invoking simple outward or inward shifts of B function gene expression boundaries to produce an entirely petaloid or entirely sepaloid perianth (**Figure2**) (Kramer *et al.*,

2003). In an elegant mechanism this model explains the occurrence of petaloidy in the outer perianth via a simple outward shift of the B gene expression domain.

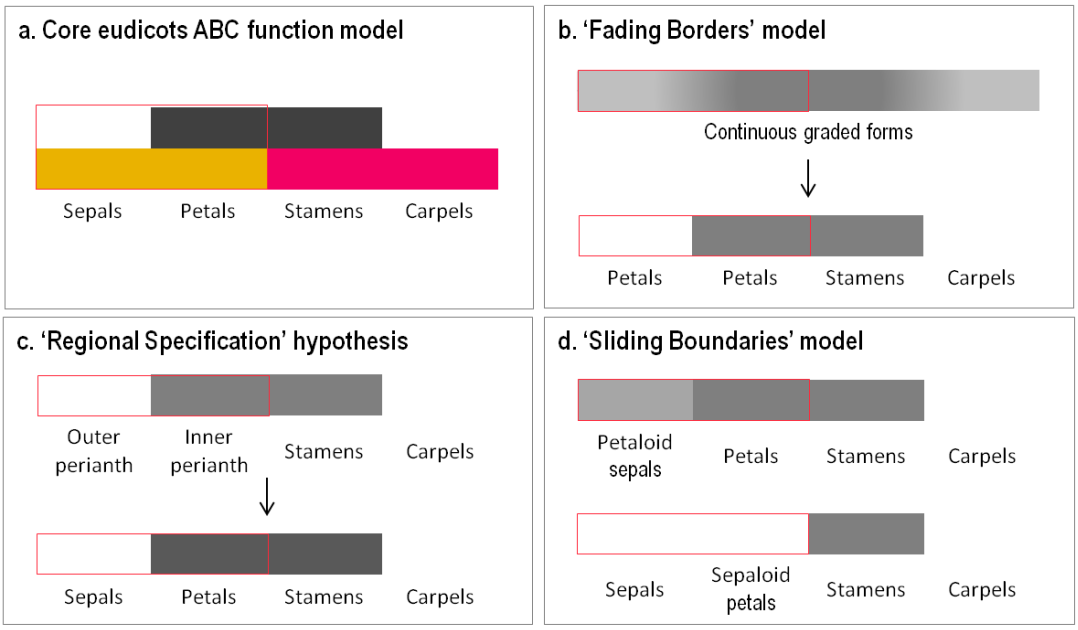

Figure 2. Summary of the different models accounting for the origin and evolution of differentiated perianths and petals. Expression of B function homologs is depicted by grey colored boxes (light to dark). Perianth is delimited by red boxes. a. A simplified depiction of the ABC model in core eudicots. Sepals are specified by the expression of A class alone (yellow) and petals by the combined action of A and B function. b. The 'fading borders' model proposed by Buzgo *et al*., 2005 views the strict floral development program with clear organ identities of higher eudicots (lower panel in b) as a secondary modification of the basal angiosperms developmentally labile progam (upper panel in b). The broad expression patterns of basal angiosperms B gene homologs are depicted by a gradient of grey while an intermediate step of gene expression canalization in depicted in the lower panel by a darker grey. c. The 'regional specification' hypothesis proposed by Irish (2009) for the ancestral role of B genes homologs as determining an inner perianth region distinct from the outer perianth and where a subsequent petal program evolved. d. A shematization of the alternative transformations to the classical ABC model B gene expression pattern postulated by the 'sliding boundaries' model to explain the origin of complex secondary floral phenotypes (presence of petaloidy in first whorl organs and production of entirely sepaloid perianth). Based on Coen and Meyerowitz 1991, Kramer *et al*. 2003, Irish 2009, Theiβen and Melzer 2007.

Both the 'fading borders' and 'sliding boundaries' models fit nicely with the 'regional specification' hypothesis that the ancestral B genes function was the specification of a regional domain, and not of a particular organ type (Ronse De Craene, 2007; Irish, 2009), as both provide an explanatory mechanism for the confinement of B gene expression to a region within the perianth without needing to involve organ identity specification roles. The origin of petals within the new boundaries of a pre-differentiated perianth would only require that these genes engage a new developmental program and/or the recruitment of new downstream genes for such functions. This model could therefore

elegantly account for both the origin of andropetals and bracteopetals, depending on whether the B function genes acquire the capacity to regulate stamen differentiation genes or genes involved in the differentiation of leaf-like aspects (Irish, 2009).

Homeosis in flowers

It is clear that transitions of a homeotic nature have played an important role in the evolution of flowers. Changes in homeotic genes are invoked in almost all modern theories of flower origin and in all current models of floral architecture diversification (discussed above). Homeotic changes could potentially arise gradually in infinitely small steps, satisfying Darwin's and the Modern Synthesis' theories. However, our understanding of contemporary homeotic mutants, particularly of the strikingly simple genetic basis that often underlies such abrupt phenotypes, makes it unlikely. Current efforts should be concerned with the confirmation of those molecular mechanisms, as well as understanding the ecological and population genetics mechanisms that have allowed for the successful establishment of such organisms and their contribution to the origin of new evolutionary lineages (Theißen, 2010). For that purpose we must stray from the classical laboratory mutants, and search for homeosis in the natural populations. Examples of research along those lines are beginning to sprout in literature, such as the study of the natural homeotic variant of *Clarkia concinna* bicalyx which involves the transformation of petals into sepal-like organs (Ford & Gottlieb, 1992), or the natural peloric mutant of *Linaria vulgaris* in which floral symmetry transitions from bilateral to radial (Cubas *et al.*, 1999). The *Capsella bursa-pastoris* '*stamenoid petals*' mutant involves as the name indicates a transformation of petals into stamens (Hintz *et al.*, 2006), and the *Vinca minor* '*flore pleno*' variant arises by partial or complete transformation of stamens into petals (Wang *et al.*, 2011). For most of these examples a remarkably simple genetic basis has been uncovered, often involving mutation at a single locus. However, the mechanisms of homeotic mutant survival in natural populations have only briefly been addressed for the majority of such examples and remain a major shortcoming of this line of research.

Mechanisms of floral traits diversification and evolution

Understanding floral architecture diversity

In order to understand the evolution of flower diversity beyond the how and the when provided by molecular, morphological and phylogenetic studies, it is indispensible to consider the functional aspects of the flower and the functional roles of the different traits. In fact, long before the development of such disciplines as molecular biology, the evolution of flower diversity already puzzled biologists. We have already seen that this was a major concern of Darwin as it directly challenged his views on gradual evolution by natural selection. When confronted with the evidence for the rapid diversification and biogeographical spread of higher plants, Darwin admitted that it could only be explained by an extremely rapid pace of evolutionary diversification or a strikingly long and missing fossil record. Unsurprisingly he dismissed the absence of fossil evidence for a long and gradual history of evolutionary transformation, which should be an indication of rapid evolutionary change, and instead chose to propose an explanation for that gap in the fossil record. He came, however, to accept an early theory that aimed to explain the seemingly abrupt and highly accelerated diversification of floral morphology, through a strong co-evolutionary interdependence mechanism between insects and flowering plants (reviewed in Friedman, 2009). Darwin's efforts to understand flowering plants diversity in light of his adaptation by natural selection theory were henceforth marked by his realization of the importance of outcrossing, and the role of insect pollination in such mating systems. The greatest consequence of this work was the promotion of the new discipline of pollination biology, which laid the basis for our current understanding of floral function and provided the most accepted hypothesis for the rapid radiation of flowering plants, that of a co-evolution with insects (Friedman, 2009; Harder & Johnson, 2009). The prevailing view, even today, is that floral diversity is the result of gradual and continuous selection accumulation in a process of adaptation of floral traits to different modes of plant reproduction, of which biotic mediated outcrossing (animal pollination) is the most effective and widespread (Fenster *et al.*, 2004; Kay & Sargent, 2009). As Stebbins (1970) put it: "The diverse floral structures and pollination mechanisms found in angiosperms represent a series of

adaptive radiations to different pollen vectors and different ways of becoming adapted to the same vector."

Pollination biology and flower diversity

The core of pollination biology theories lies in the realization of the extraordinary relationship of dependence between plants and the surrounding environment for their successful mating, i.e. production of high quality offspring via cross-pollination (Barrett, 2010; Schiestl & Johnson, 2013). Indeed, it is frequently observed that differences between major groups of angiosperms are found in association with traits implicated with reproduction efficiency and successful establishment of seedlings, whereas the vegetative aspects of plant diversity largely reflect adaptations of a same body plan to different environments (Stebbins, 1970). Despite not being exclusively necessary (self-pollinating species do exist, although not without constraints on their evolutionary potential due to their particular genetic structure), cross-pollination, that is, mating with another plant, is the norm among the majority of flowering plants reproduction systems (Stebbins, 1970). Due to their immobility plants depend either on biotic or abiotic elements for the successful transport of pollen from one plant to another. Among flowering plants the most frequently used pollen vectors are animal pollinators with which they may establish strong relations of interdependence benefitting both the plant (from a reproductive point of view) and the animal (from a foraging and nourishing point of view) (Mitchell *et al.*, 2009b). Under a comparative evolutionary context, such interactions between flowers and pollinators have given rise to the hypothesis that floral traits have adapted for pollination by different animal groups which in turn has led to convergent evolution of floral traits into common character states or syndromes. That is, that flowers exhibit a certain level of specialization for the attraction and use of specific group of animals as pollinators (Johnson & Steiner, 2000; Fenster *et al.*, 2004).

Over the past two decades the concept of "pollination syndromes" and the idea of a specialized relationship between flowers and pollinators has been challenged by the recurrent accounts of plant species that are pollinated by a numerous and widely assorted assemblage of pollinators. These

observations have led to a countering movement suggesting widespread use of generalized pollination systems. Waser and colleagues (1996) drew on theoretical models to support their observations that there is no particular association between floral phenotypes and pollinator type, hypothesizing that generalization is favored by temporal and spatial variation in pollinator communities with similar pollinating efficiencies. Other more cautionary works have suggested a continuum rather than a dichotomy (Johnson & Steiner, 2000), and some others have tried to patch up the conflicting observations of converging floral trait evolution and pollinator diversity, resorting to alternative theories on effectiveness, scale and temporal variability of pollination systems (Ollerton, 1996), the most prominent of which proposes a categorization of pollinators into functional groups rather than phylogenetic groups (Fenster *et al.*, 2004).

According to the classical view of pollination biology, by promoting intra-specific pollen transfer and reproductive isolation (or, below the species level, assortative mating between forms), specialization is intimately connected with the role plant-pollinator interactions play in plant speciation, hence in the diversification of flowering plants (Kay & Sargent, 2009). Indeed, variations in pollinator abundance and efficiency are believed to have driven major evolutionary trends in this field, such as the transition from outcrossing to selfing, from hermaphroditism to dioecy, and from animal to wind pollination (Stebbins, 1970; Barrett, 2010). However, the specialization/generalization debate has had a profound impact in pollination theories because it suggests that plant-pollinator interactions may not be specialized enough to enable the reproductive isolation conditions required for speciation (Kay & Sargent, 2009).

From a microevolutionary point of view, it is relatively easy to conceptualize the mechanisms that drive floral traits into new adaptive states, i.e. competition promoted by pollinator abundance fluctuations or by the presence of co-flowering species (Mitchell *et al.*, 2009a). The selective pressure to produce organs that are more attractive or having morphologies that promote better use of pollinators, being a form of insect-mediated selection on floral architecture that drives quantitative evolution of floral traits such as size, shape, number and reward abundance (Mitchell *et al.*, 2009b; Harder & Johnson, 2009). From a macroevolutionary standpoint, however, the mechanisms that lead

to the divergent evolution of floral traits within a species may be far more complex (Harder & Johnson, 2009; Sapir & Armbruster, 2010). Two general patterns of plant-pollinator interactions have been invoked to explain divergent evolution of flower architectures: the use of a same pollinator species, or pollinator group, in different manners by different plants within a species; and the use of different pollinators by a same species over an area of distribution (Fenster *et al.*, 2004; Kay & Sargent, 2009). While the first mechanism has been called into question by the recent lack of support for specialized relationships between plants and pollinators, pollinator shifts, that is the transition from one pollination system, or pollinator species or group, into another as a consequence of variable pollinator abundances, are believed to induce profound floral architecture modifications and promote speciation (Fenster *et al.*, 2004; Kay & Sargent, 2009). Empirical evidence for such mechanisms is still incomplete and the relative importance of plant-pollinator interactions and geographical distribution to the establishment of reproductive isolation and divergent floral evolution has been debated (Kay & Sargent, 2009). Furthermore, the mechanisms underlying pollinator shifts, that is, how a species that is potentially adapted for pollination by a certain species or group can transition adaptively to another, are still unclear (Kay & Sargent, 2009). Perhaps due to the relative low frequency of known cases of abrupt transition between distinct floral forms within a species in the wild, the relationship between pollination biology and the maintenance of mutants displaying qualitative morphological novelties at the flower level has remained, for the better part, unexplored (Harder & Johnson, 2009). Nevertheless, the potential of fundamental morphological transitions at the flower level to produce changes at pollination system level and induce divergent evolution conditions should not be ignored. In order for alternative floral forms to be strive in natural populations, they must display a certain degree of adaptiveness which is selectively advantageous (Wilson, 2011). Theoretically, this can be achieved within the same pollination environment if interactions with pollinators are not substantially changed, which is less likely the more striking the change is, or by exploring variations in pollinator communities, such as a shift in abundance of a pollinator that is more attracted or successfully used by the new form. Alternatively a shift in pollination mode, enabled by the absence of self-incompatibility issues, towards a selfing self-sufficient pollination system that doesn't require pollinators may release the new form from reproductive selective pressures during a

settling period (Wilson, 2011). Shifting from an outcrossing to a selfing pollination mode was for a long time considered to be an evolutionary irrelevant step as the impact of selfing on a species genetic structure, namely the reduced genetic variability resulting characteristic of inbreeding depression, was seen to condemn it to an evolutionary dead end (Stebbins, 1970; Charlesworth & Charleswoth, 1987). Subsequent studies, however, have demystified the dichotomy between selfing and outcrossing and shown that mixed mating mechanisms are more common than previously thought, especially in animal pollinated species (Vogler & Kalisz, 2001; Barrett, 2003). Indeed, while inbreeding depression may be a major disadvantage of selfing, several studies have also shown that selfing populations can purge their deleterious alleles reducing their genetic load and recovering normal fitness levels. Additionally, selfing can also be an advantageous strategy, particularly in a competitive pollination scenario where it can act in a reproductive assurance mechanism (Barrett, 2003; Goodwillie *et al.*, 2005).

The Nigella damascena *floral dimorphism*

Species context

This thesis deals with the rare case of a perianth composition polymorphism in wild populations of the *Nigella damascena* L. (love-in-a-mist) species. The *Nigella* genus was initially described in 1753 by Linnaeus containing six species. Today that number has been augmented to 14 species and several subspecies grouped within the Nigelleae tribe which belongs to the Ranunculoideae subfamily of the Ranunculaceae family (**Figure 3**) (Stevens, 2001 and onwards; Zohary, 1983; APG III, 2009; Wang *et al.*, 2009).

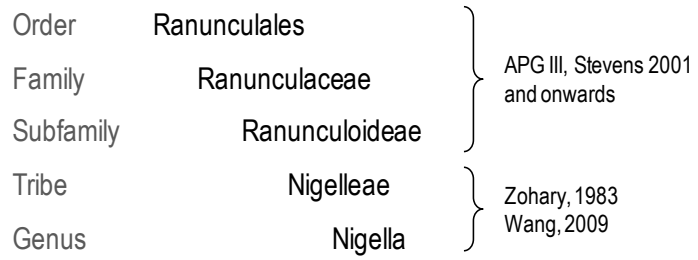

Figure 3. Taxonomic classification of the *Nigella damascena* species from order to genus according to APG III, 2009; Stevens, 2001 and onwards, Zohary, 1983 and Wang *et al*., 2009.

The geographical distribution of the *Nigella* genus members spans much of the Mediterranean areas including the western Iberian Peninsula, the north of Africa and the Near East region. Its small herbaceous plants have an annual life cycle being in the summer vegetation of semi-arid areas and typically disturbed soils. *Nigella damascena* in particular is believed to have originated in the eastern Mediterranean, likely in the Turkey, Syria and Crete regions (Zohary, 1983). In fact its specific epithet derives from the Syrian capital Damascus in reference to its likely origin, whereas the genus name *Nigella* is a reference to the dark coloration of the seeds produced by its members (Heiss & Oeggl, 2005). Around its original area of distribution the *Nigella damascena* and its relative the *N. sativa* are commonly cultivated for the medicinal properties found in its seeds which are also widely used in the eastern Mediterranean and north African cuisine as a spice and condiment (Heiss & Oeggl, 2005). In central Europe, where it has been secondarily introduced, *N. damascena* has gained a relative horticultural importance being popularly cultivated for ornamental purposes. In this region and other

regions outside its natural area of distribution, the species may only form ephemeral natural populations after escaping from cultivation (Heiss & Oeggl, 2005).

Species description

N. damascena plants share a series of characteristics with the other members of its genus, namely its highly dissected pinnatisect leaves, its hermaphrodite flowers showing a spiraled insertion of a large number of organs accompanied by the presence of a series of symmetry planes (actinomorphy) (Endress, 1999), the involucration of flower buds by the uppermost shoot leaves (bracts), the presence of conspicuous sepals and of reduced size petals bearing nectar (also called honey leaves), and the production of a capsular fruit from an inflated, partly fused, five carpel structure (Zohary, 1983).

The particularity of the *N. damascena* is the co-existence of two natural variants, composing a floral dimorphism of perianth composition (**Figure 4**). *Wild-type* flowers of the *N. damascena* as described in the species definition, have a differentiated perianth of typically five petaloid bluish sepals and eight reduced size petals consisting of a lower saccate lip bearing nectar and an upper protective scale, followed by several series of stamens and five to six carpels. This form, classically called the 'single' form will be hereafter referred to as the [P] morph for its possession of petals (Raman & Greyson, 1977; Zohary, 1983). Alternatively, the classically named 'double' variant of the *N. damascena*, here called the [T] morph after Toxopéus (1927), produces flowers with no petals but instead a large number of petaloid sepal-like organs that sit between the outer petaloid sepals and the inner stamens. In addition to the absence of petals the replacing sepal-like organs show a continuous gradient of forms, from sepals to sepal-like and sepal-like to stamens which results in an undifferentiated perianth and the production of intermediate chimeric forms at the transition between the perianth and the stamens. These intermediate organs can assume the form of bifid sepal-like organs or membranous stamens (see Chapter 1).

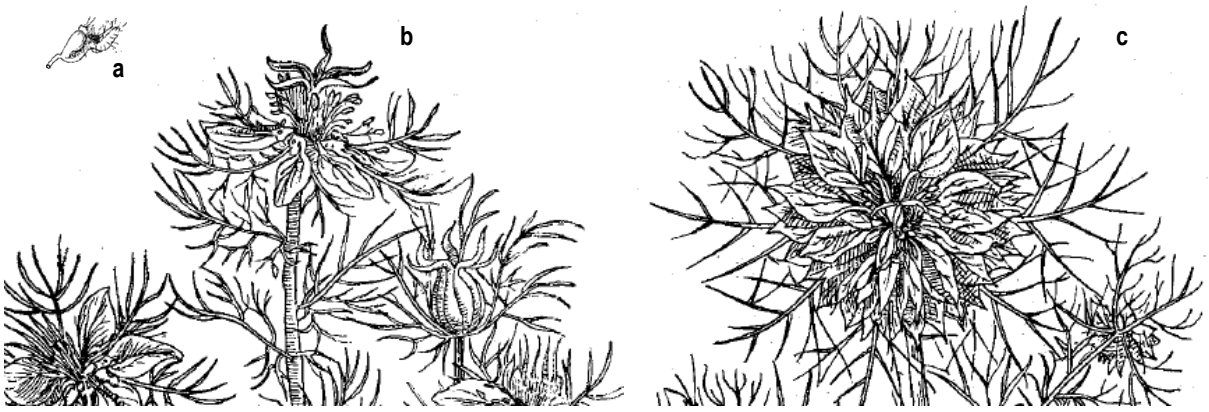

Figure 4. Illustrations of *Nigella damascena* plants producing flowers with petals (a), i.e. 'single' or [P] morph (b) and without petals, i.e. 'double' or [T] morph (c), adapted from Toxópeus, 1927 (b and c) and Zohary, 1983 (a).

In his monograph of the *Nigella damascena* species and its variants, Toxopéus (1927) showed that the floral dimorphism is monogenically controlled, with the 'single' or [P] form being dominant and the 'doubleness' or [T] morph being recessive. Accordingly the heterozygote produces a 'single' or [P] morph phenotype (Raman & Greyson, 1977). The existence of this floral dimorphism in natural populations has been reported since the first publications concerning *Nigella damascena* with several geographical accessions of mixed populations being reported across the Mediterranean region. The prevalence of the [P] morph and its shared pentamerous and tetracyclic floral traits with the rest of the *Nigella* genus members highly suggests it to be the ancestral form.

As mentioned above, the *Nigella* belongs to the Ranunculaceae family which is a member of the Ranunculales order, which is the basal-most eudicot clade, sister to all other extant eudicots (APG III, 2009). The Ranunculaceae family shows a remarkable diversity of floral morphology comprising but not restricted to a variation on the number of floral parts (merism), organ insertion (phyllotaxis) which can be spiraled, whorled or a mix of both, and most strikingly a variation of perianth form and composition (**Figure 5**). Flowers of the Ranunculaceae family can have a bipartite perianth well differentiated into sepals and petals, where sepals can often be of petaloid nature, or a unipartite perianth composed of undifferentiated organs of varied nature, sepaloid or petaloid or ranging from one to the other gradually.

Figure 5. Examples of floral diversity in the Ranunculaceae family. Top, from left to right: *Aconitum napellus*, *Consolida regalis*, *Cimifuga racemosa*, *Ranunculus abortvius*, *Trautvetteria caroliniensis*, *Pulsatilla patens*, *Adonis annua*, *Anemone hupehensis*, *Helleborus argutifolius*. Bottom, from left to right: *Caltha palustris*, *Trolius laxus*, *Aquilegia canadensis*, *Thalictrum dioicum*, *Thalictrum thalictroides*, *Xanthorhiza simplicissima*, *Hydrastis Canadensis*, *Glaucidium palmatum*. All images are from Wikimedia Commons except for *C. racemosa*, *T. dioicum*, *H. Canadensis* and *G. palmatum*. For complete credit lines see References.

The position of this family as a sister clade to the remaining eudicots makes up for a privileged working model for the study of structure and developmental processes homology in the origin of petals and evolution of perianth diversity. Combining a transition between a bipartite and unipartite perianth with an apparent loss of organ boundaries and clear differentiation, the Nigella perianth polymorphism provides unique opportunity to decipher genetic bases of perianth composition and petal formation, and to understand the ecological significance of variation in perianth form. This thesis represents a first effort into both questions.

Question 1. Molecular origin of the *Nigella damascena* floral dimorphism

The first aim of my thesis research project was to identify the molecular origin of the floral dimorphism using a candidate gene approach. At the very least a thorough study of compared gene expression dynamics between both morphs would allow for a characterization of the molecular differences associated with the perianth forms and at best a functional validation of differentially expressed genes could reveal the gene responsible for the dimorphism. Understanding the molecular mechanisms of perianth differentiation shifts is a key process in understanding the developmental and genetic bases of perianth form diversity and evolution. Identifying the key genes responsible for the presence/absence of petals also represents a first step in unraveling the genetic network controlling petal formation in basal eudicots.

Question 2. Evolutionary significance of perianth form variation

Despite possessing a set of traits likely adapted to a cross pollination reproduction mode (i.e. presence of nectaries, dichogamy and a succession of stamen and style movements that promote contact with pollinators), *N. damascena* plants are also capable of self-pollination (Zohary, 1983), which likely ensures reproductive success even in the absence of insect pollination. Nevertheless, since the morphological differences observed between the *N. damascena* floral morphs strongly affect traits that may be involved in pollinator attraction, particularly the presence of nectariferous petals and the number of potentially attractive organs, the loss of those features in the variant form is likely to affect both pollinator behavior and plant reproductive fitness.

In the second part of this project, therefore, we studied the impact of the flower dimorphism on plant-pollinator interactions and its subsequent consequences on morph reproductive success. This aim falls within the general scope of questions concerning the evolutionary significance of the floral dimorphism. Determining whether the floral dimorphism impacts pollinator attraction, reproduction mode and reproductive fitness could help predict the evolution of morph-ratio in polymorphic populations and project hypothesis for the outcome of such situations. The addressing of both these questions circles back to the subject of the initial chapters of this introduction, by presenting a unique opportunity to study the evolutionary potential of novel forms in two ways: in the strict sense, whether the apetalous [T] morph could persist in the wild and give origin to a new lineage, and in a more general sense, understanding the broad mechanisms implicated in the maintenance of alternative floral forms and the requirements for its establishment. Additionally, understanding the role of the intra-specific floral form variation in plant-pollinator interactions within this species may provide additional clues into the relative importance of such interactions in the shaping of floral diversity.

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Figure 5 references: *Aconitum napellus* by Stan Shebs CC:BY, *Consolida regalis* by BerndH CC:BY, *Cimifuga racemosa* by Dan Tenaglia, *Ranunculus abortvius* by Frank Mayfield CC:BY, *Trautvetteria caroliniensis* by ∑64 CC:BY, *Pulsatilla patens* by Jerzy Strzelecki CC:BY, *Adonis annua* by Pablo Quiles CC:BY, *Anemone hupehensis* by JJ Harison CC:BY, *Helleborus argutifolius* by Sony Mavica CC:BY, *Caltha palustris* by Jasper33, *Trolius laxus* by Derek Ramsey GFDL, *Aquilegia canadensis* JJ Harison CC:BY, *Thalictrum dioicum* Jennifer Schlick, *Thalictrum thalictroides* by Derek Ramsey GFDL, *Xanthorhiza simplicissima* CC:BY, *Glaucidium palmatum* by Ian Young.

Chapter 1. Developmental and molecular origin of the *Nigella damascena* floral dimorphism

"What I cannot create, I do not understand."

— Richard P. Feynman, theoretical physicist

Résumé en français du premier chapitre

Origine moléculaire et développementale du dimorphisme floral chez *Nigella damascena*

La première partie de ce projet de thèse a visé à déchiffrer les origines génétiques et développementales du dimorphisme floral observé chez *Nigella damascena.* Avant le début de cette thèse, Odrade Nougué (étudiante en M1) avait isolé et caractérisé au niveau moléculaire un ensemble de gènes candidats inspirés par le modèle ABC du développement floral. En conséquence, au début de la présente étude, des séquences d'ADNc partielles de quatre gènes homologues aux gènes de classe B d'*Arabidopsis* étaient connues. Ce projet a donc été divisé en trois parties. La première a porté sur l'étude comparée de l'expression des gènes candidats chez les deux formes au cours du développement floral, dont un de ces gènes (*NdAP3-3*) s'est distingué par un profil d'expression spécifique au morphe [P], et plus particulièrement une expression spécifique du pétale. La deuxième partie a été consacrée à une analyse génomique et une étude de ségrégation de polymorphismes de séquence par rapport au génotype au locus contrôlant le morphe. Ainsi, nous avons trouvé une insertion d'élément transposable au sein d'un intron du gène candidat *NdAP3-3* qui co-ségrège complète avec le locus responsable du dimorphisme. Enfin, une étude fonctionnelle basée sur le *silencing* transitoire du gène candidat *NdAP3-3*, réalisée en collaboration avec le laboratoire de Patrick Laufs (Institut Jean-Pierre Bourgin, Versailles), a conduit à sa validation en tant que locus responsable du dimorphisme floral. Ces résultats, ainsi qu'une étude plus fine de l'expression des gènes candidats au cours du développement floral (également réalisée en collaboration avec le laboratoire de Patrick Laufs), une étude parallèle du développement floral précoce du morphe mutant réalisée en collaboration avec Florian Jabbour (Muséum National d'Histoire Naturelle, Paris), et une étude préliminaire sur les effets de l'insertion de l'élément transposable sur l'expression des gènes, ont fait l'objet d'un article publié dans The Plant Journal présenté ici intégralement. Lors de la préparation de cet article, une étude parallèle sur l'origine évolutive de fleurs apétales au sein des Renonculacées a été publiée, englobant une description de la forme mutante et des patterns d'expression du gène *NdAP3-3*, ainsi que une étude de ségrégation et une description d'un allèle mutant contenant la même insertion d'élément transposable que celle décrite dans notre travail (Zhang *et al.,* 2013). Malgré leur recouvrement, les deux travaux divergent de manière significative dans l'interprétation de deux aspects des résultats : le premier sur la

relation entre l'insertion de l'élément transposable et le phénotype mutant, et le seconde sur le rôle d'*AP3-3* dans le développement floral chez *Nigella damascena* et ses implications pour l'évolution du rôle de ce gène dans la famille des Renonculacées. Ces points de divergence sont discutés dans une dernière section.

Preamble

The first part of this thesis project aimed at deciphering the genetic and developmental origins of the *Nigella damascena* perianth dimorphism. Prior to the beginning of this project, Odrade Nougué (first year master student at our lab) carried on a study to isolate and characterize at the molecular level a set of candidate genes inspired by the ABC model of floral organ identity. As a result, at the beginning of the present study, partial cDNA sequences of four genes homologous to the B class genes of *Arabidopsis* were known. This project was hence divided into three parts. The first focused on the compared study of candidate gene expression during both morphs floral development. The second part was based on genomic analysis and segregation studies of sequence polymorphisms in relation to morph. The combination of these results was expected to single out the best candidate gene which would be functionally validated in the third part. During the early stages of the gene expression study one of the candidate genes (*NdAP3-3*) stood out, showing a morph-specific, and more particularly a petal-specific, expression pattern. Subsequently we focused on this gene for the study of genomic sequence polymorphisms. We found a transposable element insertion polymorphism within an intron of this gene which was shown to co-segregate perfectly with floral morph. Finally, a functional study involving the transitory silencing of the *NdAP3-3* candidate gene, carried in collaboration with Patrick Laufs laboratory (Institut Jean-Pierre Bourgin, Versailles), led to its validation as the locus responsible for the observed floral dimorphism. These results, together with a finer study of gene expression during floral development (also performed in collaboration with Patrick Laufs laboratory), a parallel study of the mutant morph early floral development performed in collaboration with Florian Jabbour (Muséum Nationale d'Histoire Naturelle, Paris), and a brief investigation on the effects of the transposable element insertion on gene expression, were the subject of an article published in The Plant Journal. The article is presented here integrally and followed by a brief discussion of its implications as well as some perspectives into the future of this project.

Article – An APETALA3 homolog controls both petal identity and floral meristem patterning in Nigella damascena L. (Ranunculaceae)

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Summary

Flower architecture mutants provide a unique opportunity to address the genetic origin of flower diversity. Here we study a naturally occurring floral dimorphism in *Nigella damascena* (Ranunculaceae), involving the replacement of the petals by numerous sepal-like and chimerical sepal-stamen organs. We performed a comparative study of floral morphology and floral development, and characterized the expression of *APETALA3* and *PISTILLATA* homologs in both morphs. Segregation analyses and gene silencing were used to determine the involvement of an *APETALA3* paralog (*NdAP3-3*) in the floral dimorphism. We demonstrate that the complex floral dimorphism is controlled by a single locus, which perfectly co-segregates with the *NdAP3-3* gene. This gene is not expressed in the apetalous morph and exhibits a particular expression dynamic during early floral development in the petalous morph. *NdAP3-3* silencing in petalous plants perfectly phenocopies the apetalous morph. Our results show that *NdAP3-3* is fully responsible for the complex *N. damascena* floral dimorphism, suggesting that it plays a role not only in petal identity but also in meristem patterning, possibly through the regulation of perianth organ number and perianth-stamen boundary.

Key words: Perianth architecture, B-function genes, petal identity, floral meristem patterning, homeotic transformation, Ranunculaceae, *Nigella damascena*

Introduction

The flower is very likely the structure that most contributed to the angiosperms evolutionary success. It typically consists of two inner whorls containing the male and female reproductive organs, surrounded by two outer whorls of sterile organs, the sepals and petals, which collectively form the perianth and often serve as attractive structures for insect-pollinated species. Variations in the composition and shape of perianth contribute greatly to the remarkable diversity of flower architectures observed across the angiosperms, the origin of which has intrigued developmental and evolutionary biologists for almost two centuries (Friedman and Diggle, 2011). The detailed study of floral homeotic mutants in the core eudicot species *Arabidopsis thaliana* and *Antirrhinum majus* led to the proposal of a genetic model for floral organ identity specification (Bowman *et al.*, 1991; Coen and Meyerowitz, 1991). According to this model, three classes of genes (A, B and C) act in concentric overlapping fields within the floral meristem to specify the identity of the four floral organs: A alone specifies sepals, A+B petals, B+C stamens and C alone carpels. Since its description more than 20 years ago, this model has been expanded, as new gene classes have been discovered (Angenent *et al.*, 1995; Pelaz *et al.*, 2000), and reappraised, as new studies outside the core eudicots reveal the extent of its conservation across the angiosperms. Notably, expression studies of the different classes of organ identity genes in basal eudicot and basal angiosperm flowers have revealed a broad consistency with the predictions of the ABC model, particularly in the B-and C-class genes (D., E., Soltis *et al.*, 2007; Theissen and Melzer, 2007; Litt and Kramer, 2010).

In *Arabidopsis* the B function is fulfilled by the MADS-box genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Bowman *et al.*, 1989; Krizek and Meyerowitz, 1996). *AP3* and *PI* belong to two related gene lineages issued from an ancient gene duplication which likely occurred before angiosperm diversification. Following this ancient duplication, the two gene lineages were subjected to different evolutionary histories across the angiosperms (Kramer *et al.*, 1998). In the basal eudicot order Ranunculales two additional gene duplications took place in the *AP3* lineage, the last one after the divergence of the Papaveraceae, resulting in the production of three *AP3* paralogs (*AP3-1*, *AP3-2* and *AP3-3*) (Kramer *et al.*, 2003; Rasmussen *et al.*, 2009; J., Hu *et al.*, 2012). The retention of these *AP3*

paralogs has been attributed to gene subfunctionalization and/or neofunctionalization, which could account for the divergent expression patterns observed among them and the possible evolution of novel regulatory functions in organ identity specification (Kramer *et al.*, 2003; Rasmussen *et al.*, 2009; Stellari *et al.*, 2004). Indeed, while the *AP3-1* and *AP3-2* paralogs have broad and temporally variable expression domains, the *AP3-3* paralog exhibits a petal-specific expression and an even more remarkable absence of expression in apetalous species, which makes it a likely candidate for the petal identity specification function (Rasmussen *et al.*, 2009; R., Zhang *et al.*, 2013). Despite this general correlation, the involvement of *AP3-3* in the petal identity program has only been functionally validated in the petalous Ranunculaceae species *Aquilegia coerulea*, in which *AP3-3* gene silencing led to the conversion of petals into sepals (Sharma *et al.*, 2011). Additional functional studies are needed in order to determine whether the *AP3-3* role in petal identity specification and perianth architecture is conserved on a wider scale.

The Ranunculaceae family, the richest in species among Ranunculales, exhibits a remarkable diversity of perianth architecture, displaying numerous transitions between a bipartite perianth, possessing morphologically differentiated sepals and petals, and a unipartite perianth, consisting of undifferentiated organs either entirely sepaloid or entirely petaloid. The *Nigella damascena* L. (love-in-a-mist) presents a rare case of a bipartite to unipartite perianth transition at the species level, resulting in a perianth architecture dimorphism. This unipartite morph has been previously described as a double-flower mutant, lacking petals and instead having a series of sepal-like organs (Toxopéus, 1927). Studies on the organ identity shift have revealed its monogenic control by a bi-allelic locus, with the petalous form being dominant (Toxopéus, 1927), as well as the tight association of the *NdAP3-3* paralog with the petalous form, both at the expression pattern and genomic sequence levels (R., Zhang *et al.*, 2013).

Here we describe and investigate the genetic and molecular origin of a previously disregarded aspect of the *Nigella damascena* perianth dimorphism, involving an increase in total perianth organ number and the production of a gradation of organ morphologies from entirely sepal-like to mixed sepal and stamen-like, suggestive of a disruption of the boundary between perianth and reproductive organs. We

characterized in detail the floral morphology and floral development of both morphs and performed a comparative analysis of the *APETALA3* and *PISTILLATA* homologs gene expression. Segregation analysis and virus induced gene silencing (VIGS) studies provide compelling evidence for the involvement of the *NdAP3-3* paralog in all the aspects of the floral dimorphism. We show that *NdAP3-3* plays a dual role in flower development, determining not only the identity of the petals, but also controlling floral meristem patterning through the regulation of perianth organ number and the proper establishment of a perianth-stamen boundary. We discuss this dual role in an evolutionary context within the Ranunculaceae.

Results

The *Nigella damascena* floral dimorphism encompasses not only a shift in petal identity but also in perianth organ number and in the perianth-stamen boundary

Two *Nigella damascena* L. floral morphs were observed among the plants sown in greenhouse from a sample of seeds collected in the natural population of Mornas. The most commonly observed form, hereafter the [P] morph, has four organ types inserted in a spiral arrangement: about five sepals and eight petals, a variable number of stamens arranged in eight parastichies and a gynoecium of five proximally connate carpels (Fig. 1a). Mature sepals have an ovate simple blade with a lanceolate apex and a range of petaloid characteristics such as a bright blue coloration and the presence of papillated striated conical cells on the adaxial surface (Fig. 1c,i). On the abaxial side cells are irregularly shaped, pavement-like and interspersed with stomata (Fig. 1j). Petals are small dark blue organs with a narrow stalk-like base, two apical lobes bearing two round glistening pseudonectaries, and a nectariferous pouch covered by a flat scale (Fig. 1d). The petal cellular epidermis is composed of papillated conical cells with ornamentations and interspersed with trichomes on the adaxial side of the apical lobes (Fig. 1k), and regular elongated ornamented cells on the entire abaxial side as well as on the stalk and the adaxial surface of the nectary operculum (Fig. 1l). Flower size and total perianth organ number may vary within an inflorescence according to flower position.

The less commonly observed floral morph in the Mornas population, hereafter the [T] morph, resembles the double-flowered variant described by Toxopéus (1927). While the five sepals in [T] morph are equivalent in structure and cellular composition to those found in the [P] morph (Fig. 1e), the nectariferous petals are absent and are replaced by a variable number of sepal-like organs (Fig 1b). These include a range of shapes from outermost lanceolate entirely sepaloid (Fig. 1f), to intermediate organs with bifid or trifid apices (Fig. 1g), to innermost hybrid organs (Fig. 1h) that have mixed sepal and stamen characteristics such as a thin filament-like stalk and a half-sporangiate half-sepaloid apical blade. Cellular composition in the inner perianth organs resembles the sepal epidermis with conical cells on the adaxial side and pavement cells interspersed with stomata on the abaxial surface. The

inner stamens as well as the carpels of the [T] morph flowers are similar to those described above for the [P] morph. In addition to the production of sepal-stamen hybrid organs in [T] morph, there is a significant increase in total perianth organ number. Indeed, the number of perianth organs in [T] morph (sepals + sepal-like) is significantly greater than that of the [P] morph (sepals + petals) (mean ± sd: [P] 12.88 ± 1.02, [T] 23.42 ± 5.06, $P < 2.2x10^{-16}$). Thus, although the loss of petals and production of sepal-like organs in their place might resemble a classical homeotic transformation, the *N. damascena* perianth polymorphism case is more complex, implying a shift in perianth organ number and perianth-stamen boundary disruption.

Perianth organ identity, perianth organ number and boundary shift are determined by a single locus

The *N. damascena* flower dimorphism studied by Toxopéus (1927) is determined by a single locus, the petalous form being dominant over the apetalous one. We named this the P locus, and the dominant allele responsible for the presence of petals the *P* allele, whereas the homozygous *pp* combination produces no petals. In our observations, organ identity transformation and the shift in organ number were always associated in the [T] morph. In order to confirm the monogenic determinism not only of the organ identity shift but of the whole complex flower phenotype, we analyzed three F2 populations segregating for floral morph. We confirmed that the [P] and [T] proportions do not differ significantly from the 3:1 ratio expected in a dominance scenario (97 [P] to 41 [T], $P = 0.1941$). Furthermore, the shift in petal identity, the presence of supplementary sepal-like organs and disruption of the perianth-stamen boundary were always associated and observed only in [T] morph. Test-cross of F2 [P] plants indicated that there is a 2:1 ratio of heterozygous to homozygous plants (76 *Pp* to 21 *PP*, $P = 0.0146$), confirming the monogenic dominant nature of the flower dimorphism determinism which encompasses not only perianth organ identity but also perianth organ number and perianth-stamens boundary regulation.

Compared floral development in [P] and [T] morph

During floral organogenesis in the [P] morph, four different types of primordia are initiated in a centripetal way: crescent-shaped and fast growing; hemispherical with a break in the development; hemispherical without a break in the development; and uppermost horseshoe-shaped (Fig. 2a-d, (Zhao *et al.*, 2011; Jabbour *et al.*, 2009)). These four types of primordia will become sepals, petals, stamens, and carpels, respectively. In the present study, we broke down the early development of [P] morph floral buds into four stages. The first stage (S-I) corresponds to calyx initiation (Fig. 2a). During S-II, primordia of petals and lowermost stamens initiate and are indistinguishable in shape and size (Fig. 2b). At stage S-III, petal primordia display a particular flattened shape and their development is delayed, while the remaining stamens are initiated and their development continues (Fig. 2c). S-IV is characterized by carpel initiation (Fig. 2d). By this time petal and stamen primordia are easily distinguishable by their shape and differentiation state.

In contrast to the [P] morph floral development, only three visibly different types of primordia are initiated during [T] morph organogenesis, namely, in a centripetal way: crescent-shaped and fast growing, crescent-shaped to hemispherical and uppermost horseshoe-shaped (Fig. 2e-h). These three types of primordia will become sepals, sepal-like organs or stamens, and carpels, respectively. The preanthetic development of [T] morph was similarly broken down into four stages. S-I corresponds to calyx initiation (Fig. 2e). During S-II undifferentiated future sepal-like organ and stamen primordia are initiated (Fig. 2f). During the next stage (S-III) the outermost of these primordia become crescent-shaped and develop in a sepal-like way (outermost in Fig. 2g), while the youngest innermost primordia, remain indistinguishable. Stage S-IV is marked by the initiation of carpel primordia (Fig. 2h). By this stage a good proportion of earlier initiated primordia can be identified as sepal-like organs but, as the innermost perianth organs share stamen-like characteristics, the identity of inner primordia and the point beyond which true stamen primordia begin cannot be fully discerned yet.

Compared expression patterns of *APETALA3* and *PISTILLATA* homologs in [P] and [T] morph

We investigated the *Nigella damascena APETALA3* and *PISTILLATA* homologsexpression patterns in [P] and [T] developing floral organs from stages >IV using RT-PCR. Our results confirm the previously observed petal-specific pattern expression of the *NdAP3-3* paralog in the [P] morph floral development, and its complete absence from developing [T] perianth organs (Fig. 3, (R., Zhang *et al.*, 2013)). Although a qualitative difference in expression could be detected for the *NdAP3-3* paralog, no significant differences in the *NdAP3-1*, *NdAP3-2* and *NdPI* genes expression patterns could be observed (Fig. 3). The expression patterns of all four genes were further investigated during early flower development using *in situ* hybridization.

In [P] morph, *NdAP3-1* expression can be detected broadly at stage S-I (Fig. 4a). Later on, this expression becomes restricted to stamen primordia (Fig. 4b-d). *NdAP3-2* expression is not detected at stage S-I, but only later upon petal and stamen primordia initiation at stage S-II. It first appears in these organs primordia where it persists throughout the floral development (Fig. 4e-h). Expression of *NdAP3-3* is first detected in a region of the undifferentiated floral meristem that encompasses the sites of the future petal and stamen primordia (Fig. 4i). This expression persists as development proceeds but becomes restricted to petal primordia at stage S-IV (Fig. 4j-l). Transcripts of *NdPI* can be strongly detected in the early floral meristem and in sepal, petal and stamen developing primordia, but not in the future carpel primordia region (Fig. 4m-p).

In accordance with the RT-PCR results, *in situ* hybridization for *NdAP3-1*, *NdAP3-2* and *NdPI* in developing [T] floral meristems revealed mostly comparable expression patterns to those described for the [P] morph. While the *NdAP3-1* paralog is absent from both developing petals and sepal-like organs (Fig. 5b-d), and *NdPI* is similarly expressed in inner perianth organ primordia and stamens of both morphs (Fig. 5m-p), *NdAP3-2* is expressed in [P] developing petals but absent from the apetalous developing perianth of [T] buds (Fig. 5f-h). Most remarkably, *NdAP3-3* transcripts could not be detected at any stage in [T] floral buds, indicating that this paralog is not expressed at all in this morph (Fig. 5i-l). The striking qualitative difference in *NdAP3-3* expression patterns between the [P] and [T]

morphs is highly suggestive of a specific role for this gene in the observed *N. damascena* perianth morphologies.

A MITE insertion in the *NdAP3-3* locus perfectly co-segregates with the P locus

The comparison of the genomic structure and sequence of the *NdAP3-3* locus from one [T] and [P] plants from each available accession revealed no polymorphisms except for a 250 bp insertion in the second intron, which was homozygous in all [T] plants and either heterozygous or absent in [P] plants. This insertion bears all the characteristics of a type II non-autonomous transposable element, i.e. short size, terminal inverted repeats (TIR) and target site duplication (TSD). Based on these structural features this transposable element is most likely a MITE (Miniature Inverted repeat Transposable Element). We investigated the segregation pattern of this insertion in the three F2 populations segregating for the flower morph. In all 136 plants, the insertion perfectly co-segregated with floral morph and genotype at the P locus (Supplementary Table S1). These results confirm the *NdAP3-3* as a good candidate for the P locus or suggest it is in very close proximity to the responsible gene.

We investigated the possibility of an altered splicing pattern induced by the MITE presence, in the [T] morph. Using different primer couples and enhanced PCR we were able to detect in very low levels, two alternative transcripts in [T] floral cDNA, containing either a 3' fragment of the second intron or the whole second intron including MITE (Supporting information Fig. S1). No wild-type transcripts could be found in this morph.

Functional validation of *NdAP3-3*

In order to demonstrate the role of *NdAP3-3* in perianth organ identity and floral meristem patterning, we used a TRV-based VIGS method to reduce its expression in [P] plants. Similarly to previous studies, we used a TRV2 construct containing a fragment of the *ANTHOCYANIDIN SYNTHASE* (*ANS*) gene which allows for an easy identification of effective silencing. Treatment with TRV2-*ANS* alone did not affect flower development or organ identity (Fig. 6a) but generated an array of *ANS* silencing phenotypes that varied according to the timing of infection. In late and intermediate inoculations, it led

to completely white sepals and green petals (Fig. 6a) or big white sectors in sepals and green sectors in petals. In contrast, flowers from early inoculations showed only small sectors of white or green in sepals and petals respectively, or a light dotted-like discoloration (as seen in Fig. 6b and c), indicating that the efficiency of *ANS* silencing increased with the time of inoculation. On the contrary, the effect of *NdAP3-3* specific silencing decreased with the time of infection. For this reason we did not refer to *ANS* silencing phenotypes as a strict guide in detecting *NdAP3-3* silenced flowers, but rather to their organ morphology and organ number, and the *a priori* knowledge of the [T] morph perianth architecture. Expression analysis of presumed transformed organs was used to confirm complete silencing. In addition to the customary VIGS controls we also performed parallel inoculations in [T] morph plants. *ANS* silencing phenotypes in [T] perianth organs were comparable to those observed in [P] plants. *NdAP3-3* silencing had no effect on [T] perianth morphology and composition, as was expected since no *NdAP3-3* transcripts can be found in this morph, suggesting that silencing was specific to the *AP3-3* paralog.

Effect of *NdAP3-3* silencing on perianth organ identity

Upon *NdAP3-3* silencing, sepals were unaffected and petals showed a range of organ identity transformation phenotypes into sepal-like organs (Fig. 6d-g). Petals on late and intermediate inoculated plants were partially transformed, keeping the same overall shape with a reduced operculum and semi-fused apical lobes in some flowers (Fig. 6b,e), or having an elongated blade with a residual nectary, almost or completely fused lobes and no pseudonectaries in others (Fig. 6f). All these semi-transformed petals already showed a transformation of cellular identity, having typical sepal cells on both the adaxial and abaxial side (Fig. 6h,i). Additionally, these organs do not express *NdAP3-3* but express *NdAP3-2*, which can be used as a proxy for the switch from petal to sepal identity as this pattern is specifically detected in the mature sepal and sepal-like organs (Fig. 7). Finally, early *NdAP3-3* silencing led to complete morphological transformation of petals into sepal-like organs with elongated lanceolate single blade, no signs of the fused lobes, nectary crease or

operculum (Fig. 6g). Like the semi-transformed organs, completely transformed petals have a complete cellular and molecular sepal identity (Fig. 7).

Effect of *AP3-3* silencing on perianth organ number

In early silenced plants, where all petals showed complete morphological transformation, some flowers also showed supplementary perianth organs. This led perianth organ number to values higher than control [P], which overlapped the normal [T] morph values (Fig. 6c and Fig. 8). The increase in perianth organ number was accompanied by the production of hybrid organs with mixed sepal and stamen characteristics, indicating a disruption of the perianth-stamen boundary (Fig. 6c). These results confirm that the *NdAP3-3* locus alone is responsible for the complex dimorphism, being implied not only in the petal identity program but also in the control of perianth organ number and perianth boundary with reproductive organs.

Discussion

Polymorphisms affecting flower architecture despite being seldom observed in nature, may have a significant evolutionary potential in the generation of species diversity, provided their impact on reproductive fitness does not significantly decrease the selective value (Hintz *et al.*, 2006; Theissen, 2006). Such polymorphisms also provide valuable insight into the genetic origin of flower architecture. The naturally occurring *Nigella damascena* mutant we describe here falls in this category. It exhibits a complex phenotype encompassing not only a change in the identity of inner perianth organs, but also an increase in total perianth organ number and a production of novel organ morphologies that suggests a disruption of the perianth-stamens boundary. Using segregation analysis and gene silencing, we provide compelling evidence that a single gene, the *NdAP3-3* paralog, is responsible for all aspects of this phenotype.

Monogenic control of the dimorphism and co-segregation with a MITE insertion at the *NdAP3-3* locus

The complex *N. damascena* perianth phenotype of the apetalous morph is remarkably different from the classical B-function homeotic mutants described in core eudicot model species where petals and stamens are replaced by sepals and carpels respectively (Jack *et al.*, 1992; Sommer *et al.*, 1990), and from other basal eudicot models, where petals are replaced by sepaloid organs upon B-class gene inactivation but organ number is unaffected (Kramer *et al.*, 2007; Sharma *et al.*, 2011; Drea *et al.*, 2007). Using segregation analysis, we confirmed that petal identity and perianth organ number are controlled by the same locus. A miniature inverted transposable element (MITE) inserted in the *NdAP3-3* paralog was found to co-segregate completely both with petal identity (this study and Zhang *et al.*, 2013), perianth organ number and perianth-stamen boundary regulation (this study). Interestingly, the same *NdAP3-3* mutant allele was found in all genetic origins we studied, as well as in those studied by Zhang *et al.* (2013), suggesting that a single mutation event was at the origin of the *N. damascena* dimorphism. The MITE insertion has been suggested to be the cause of the absence of expression (R., Zhang *et al.*, 2013), however, although these transposable elements have been

preferentially found in the vicinity of plant genes, there is little compelling evidence for their function on gene regulation (Casacuberta and N., Santiago, 2003; Feschotte, 2008; Wessler *et al.*, 1995). An alternative hypothesis is that the MITE insertion is simply a consequence of a pseudogenization process after an initial mutation, likely in a regulatory sequence.

Comparative floral development

Organ initiation, phyllotaxy, and growth pace were previously described for the petalous *N. damascena* morph by Jabbour *et al.* (2009) and Zhao *et al.* (2011). Therefore, we chose to focus our developmental study on the short time window when petals and stamens, and sepal-like organs and stamens are initiated and develop in the [P] and [T] morphs, respectively. While petal and stamen primordia in [P] morph floral buds appear morphologically identical at initiation, there is soon thereafter a delay in the developmental rate of petals, characterized by a flattening of the primordia. This petal-specific developmental delay makes it possible to identify them with certainty while sepals and stamens progress in their development. Both the early morphological similarity between developing petal and stamen primordia, and the subsequent delay in petal development have been previously identified and described during floral development in several species of Ranunculaceae (Jabbour *et al.*, 2009; Zhao *et al.*, 2011, and references therein; Kosuge, 1994).

Additionally, we present a detailed developmental study of the [T] floral morph. At early stages, all developing inner perianth organ primordia are morphologically identical and inserted on the same ontogenic spiral. Morphological differences gradually arise as primordia develop. However, because of the similarities between the innermost sepal-like organ primordia and the outermost true stamen primordia, it is impossible to determine the physical limit in the ontogenic sequence between perianth and stamen primordia. This situation is reflected in the corresponding adult flower, in which the boundary between perianth and stamens is blurry as revealed by the occurrence of hybrid organs.

NdAP3-3 is responsible for the complex floral dimorphism through a dual role in petal identity and floral meristem patterning

In order to functionally validate the *NdAP3-3* candidate as the locus responsible for the perianth dimorphism, we conducted a VIGS experiment at three different time periods relative to floral transition. Silencing specificity was confirmed by the absence of effect on [T] morph flower development and morphology, and on the expression levels of other *AP3* paralogs. Depending on the timing of gene silencing, different degrees of floral transformation were observed. While intermediate to late silencing only affected petal identity, early *NdAP3-3* silencing resulted in petal transformation into a range of sepal-like organs and an increase of the total perianth organ number, revealing a dual role of *NdAP3-3* in petal identity specification and floral meristem patterning. This dual role parallels the particular expression pattern dynamics of *NdAP3-3* uncovered during early [P] flower development.

At early stages of floral development, our *in situ* hybridization study revealed a broad expression domain of *NdAP3-3*, encompassing not only the region of future petal primordia but also adjacent upper regions that will give rise to the stamen primordia. We hypothesize that this early broad expression pattern of *NdAP3-3* reflects a role in meristem patterning, defining a "non-sepal" morphogenetic domain and controlling perianth organ number, possibly via a cell proliferation repression function. In Arabidopsis, *AP3* is rather involved in cell proliferation promotion (Krizek and Meyerowitz, 1996), and a direct role of an *AP3* homolog in cell proliferation repression has not yet been reported.

The early *NdAP3-3* broad expression domain and the potential role in domain specification and regulation of organ number relates well to the hypothesis of a regional specification function that has been put forward for the ancestral *AP3/PI* genes (Drea *et al.*, 2007). This ancestral function was proposed to account for the broad and dynamic expression domains of *AP3/PI* and their ability to specify both petaloid and non-petaloid inner perianth organs in basal angiosperms (Drea *et al.*, 2007; Irish, 2009). Under this hypothesis, the absence of *NdAP3-3* expression could lead to the disruption of the perianth-stamen boundary simply by releasing the regional specification program constraint and

allowing sepal and stamen identity programs to pervade the unregulated middle ground. The overlapping of sepal and stamen identity programs could then lead to the production of the transitional hybrid forms with mixed organ identities, much like the mechanism proposed in the fading borders model (Buzgo *et al.*, 2004; Buzgo *et al.*, 2005; Kim *et al.*, 2005). This model is based on the observation of the broad and overlapping expression domains of floral organ identity genes in basal angiosperms, which coincide with the presence of gradual transitions between organ forms, from sepals to petals and petals to stamens in flowers with spiral phyllotaxy (Kim *et al.*, 2005). Alternatively, the boundary disruption in the absence of *NdAP3-3* expression could be the result of a more direct role of this paralog in a cadastral mechanism, restricting C gene expression to the central region of floral meristem. While in core eudicot models this antagonistic role is performed by A-function genes, no A-class homologs have yet been found in Ranunculaceae (Litt and Irish, 2003). *NdAP3-3* might perform this function in a mechanism similar to the one described in Arabidopsis, where *AP3* activates the cadastral gene *SUPERMAN* at the boundary between stamens and carpels (Bowman *et al.*, 1992), but here at the petal-stamen boundary.

The complete transformation of petals into sepal-like organs, both in morphology and cellular composition, is consistent with a role of *NdAP3-3* in petal identity specification and morphogenesis. A petal identity role for the *AP3-3* orthologs within the Ranunculaceae family has been strongly suspected based on expression pattern analysis in other petalous species, but it has only been validated in *Aquilegia* so far (Sharma, Rasmussen *et al.*, 2009). The restriction of *NdAP3-3* expression domain to petal primordia at the time of their developmental delay might reflect a cell proliferation inhibition function at the onset of the petal identity program, analogous to the one put forward for the regional specification role. The sustained *NdAP3-3* petal specific expression throughout petal development supports an additional role in petal morphogenesis and cellular identity specification.

A particular dynamic is evidenced between the petal identity and meristem patterning *NdAP3-3* roles by the early and late silencing effects. We followed [P] plants that showed complete silencing phenotypes until untransformed flowers reappeared, indicating later reactivation of *NdAP3-3* due to the transient nature of VIGS. In this time window, flowers with organ number transformation but no

organ identity transformation (i.e. supplementary petals) were never observed. The absence of such phenotypes indicates an irreversibility of early *NdAP3-3* silencing, and a dependency of its function in petal identity on its own early expression. This could be either because the petal identity program is dependent upon the floral meristem patterning role, or because *NdAP3-3* expression is dependent on its own downstream targets to be sustained in a feedback loop mechanism.

Evolutionary perspectives on the *NdAP3-3* dual role

An interesting issue to be considered is whether this dual role in floral meristem patterning and petal identity specification is specifically derived in *Nigella damascena* or perhaps more largely distributed among Ranunculaceae. While a number of studies support an ancestral role of *AP3-3* genes in petal identity specification (Rasmussen *et al.*, 2009; R., Zhang *et al.*, 2013), the lack of previous report on floral patterning function leaves its evolutionary origin an open question. In *Aquilegia vulgaris*, the only other Ranunculaceae species to have been studied in detail, the *AP3-3* paralog expression appears to be restricted to petal primordia throughout floral development (Kramer *et al.*, 2007). Functional studies using *AP3-3* specific silencing in the petalous *Aquilegia coerulea*, led to the transformation of petals into sepals, without affecting the development of other floral organs (Sharma *et al.*, 2011). Interestingly, whereas in *Nigella* all floral organs are inserted in a spiral, in *Aquilegia* only sepals are spirally inserted while petals and stamens are whorled (Tucker and Hodges, 2005). The partly different consequences of *AP3-3* absence of expression in the two species could reflect different developmental constraints imposed by the spiral *vs.* whorled phyllotaxy. Another possibility is that *AP3-3* plays no role in floral meristem patterning in *Aquilegia*. Because functional studies of the *AP3-3* paralog have only been made in two species, it is presently impossible to distinguish between a scenario in which the floral meristem patterning function was lost in *Aquilegia*, or specifically acquired in *Nigella*. Unipartite perianths evolved several times independently from bipartite ancestors within the Ranunculaceae family (Rasmussen *et al.*, 2009). Such events have been shown to occur in association with an absence of *AP3-3* expression (R., Zhang *et al.*, 2013). However, few cases of transitional organs have been described (Ronse de Craene and Brockington, 2013), questioning the involvement of

this gene in the regulation of the perianth-stamen boundary. Resolving such issues requires studying additional Ranunculaceae species for the expression and function of *AP3-3* in a phylogenetic framework, in parallel with the phylogenetic mapping of evolutionary transitions between spiral and whorled phyllotaxy, and between bipartite and unipartite perianth in relation to the presence or absence of transitional organ forms.

Experimental Procedures

Plant Material

Plant origin: *Nigella damascena* L. plants having flowers with and without petals (respectively [P] and [T] floral morphs) originated from a natural population in Mornas (Vaucluse, South of France, accession 04-98 of the French National Museum of Natural History collection), and three cultivated commercial seed lots (Royal Fleur, Truffaut and Vilmorin).

Production of a segregating population: Three F2 segregating populations were produced by selfing heterozygous [P] plants issued from commercial seed lots. Based on the monogenic dominant morph determination (Toxopéus, 1927), [T] F2 plants were considered of *pp* genotype. One flower from each [P] F2 plant was test-crossed using [T] pollen and the flower morph of eight offspring per test-cross was used to determine the original F2 [P] plant genotypes (either *PP* or *Pp*). Fresh F2 plant material was sampled for DNA extraction and genotyping as described below.

Culture conditions: Plants were grown in greenhouse under long day period (16h day – 8h night) at 21ºC during the day, 17ºC during the night and 60% relative humidity.

Floral morphology and SEM floral development study

Flowers at anthesis were observed in greenhouse and photographed with an Olympus E410 camera (Japan). Individual organs were dissected from mature flowers and photographed using the Axio ZoomV16 stereomicroscope system (Carl Zeiss, Germany). Floral buds from successive developmental stages were sampled, fixed in FAA (85 ml 55 % ethanol, 5 ml glacial acetic acid, 10 ml formaldehyde) and stored in 70 % ethanol. Buds were dissected under a MZ6 stereomicroscope (Leica Microsystems, Germany), dehydrated in an ethanol series, and dried with an Emitech K850 critical point dryer (Quorum Technologies, UK). Dried floral structures were mounted on aluminium stubs with colloidal graphite, sputter coated with gold using a JFC-1200 fine coater (JEOL, Japan), and observed with a JSM-840A scanning electron microscope (JEOL, Japan) or a SU3500 scanning electron microscope (Hitachi High-Tech, Japan).

Candidate gene characterization

During the course of our study, partial coding sequences for the three *Nigella damascena AP3* paralogs, as well as the genomic sequence for the *NdAP3-3* locus have been published (R., Zhang *et al.*, 2013). We obtained similar sequences for the three *AP3* paralogs and the *PI* homolog using classical degenerate primer PCR and 5'- and 3'-RACE PCR methods described in Supplementary Material.

Expression analysis

RT-PCR: Floral buds ranging from 5 to 6 mm in diameter were dissected into sepals, petals, stamens and carpels for [P] buds, and sepals, sepal-like organs, stamens and carpels for [T] buds. Two biological repeats for each morph were prepared each pooling approximately 10 dissected buds for each morph. Total RNA was extracted using the Qiagen RNeasy kit following manufacturer instructions. DNase treatment was performed with Ambion DNaseI (Invitrogen) and single stranded cDNA was produced using SuperScriptII reverse transcriptase (Invitrogen) and random hexamers (pdN6) (Damerval *et al.*, 2007). Possible DNA contamination was excluded by performing no-RT negative controls using *ACTIN* specific primers. Each gene was amplified with specific primers (all listed in Supplementary Table S2). *ACTIN* was also used as a reference for cDNA quantity calibration among samples.

***In situ* hybridization:** [P] and [T] flower buds were sampled at a range of developmental stages and fixed under vacuum in freshly prepared PFA (4% paraformaldehyde). Dehydrated tissues were embedded in Paraplast Plus (McCormick Scientific) and sectioned to 8 µM (Damerval *et al.*, 2007). Digoxigenin-labeled RNA antisense probes were synthesized from cDNA with T7 RNA polymerase (Riboprobe System T7, Promega) using primers listed in Table S2. Probes longer than 250 bp were hydrolyzed. Slide pre-treatment, pre-hybridization and hybridization were performed as described by Damerval *et al.* (2007). Hybridized sections were digitally photographed using a ProGres C10 camera (Jenoptik, Germany) mounted on a Microphot-FXA microscope (Nikon, Japan). Photomicrographs contrast and brightness was adjusted using ImageJ and Gimp softwares.

VIGS

The TRV1 and TRV2 vector containing an *Aquilegia vulgaris ANHOCYANIDIN SYNTHASE* sequence (*AqvANS*) were kindly provided by E. Kramer (Kramer *et al.*, 2007). A 305 bp fragment specific to the *NdAP3-3* locus was amplified from the 3' cDNA and UTR using primers designed to add a BamHI and a SacI restriction sites at the 5' and 3' ends, respectively (5'-GGATCCTGGACATTACAATTTACGACTGG, 3'-AGCTCTCCCAAACAAGGTCTACTTAATCCC). This PCR product was purified and cloned using the pGEM-T Easy Vector system (Promega). The fragment was excised by double digestion with BamHI/SacI and purified before ligation into a similarly digested TRV2-*AqvANS* construct. The TRV2-*AqvANS-NdAP3-3* construct was transformed into *Escherichia coli*, positive clones were verified by PCR amplification and plasmid was extracted for transformation into *Agrobacterium tumefaciens* strain GV3101. Separate liquid cultures for each construct (TRV1, TRV2, TRV2-AqvANS and TRV2-*AqvANS-NdAP3-3*) were grown overnight and cells were collected by centrifugation before resuspension in infiltration buffer at a 2.0 final OD (10 mM MES, 10 mM $MgCl_2$, 100 mM acetosyringone). TRV1 solution was mixed with each of the other three solutions in equal volumes and incubated for 3 hours on ice prior to infiltration.

Three series of about 42 [P] and 28 [T] plants issued from the selfing progeny of homozygous plants in the F2 segregating populations were used in three assays (early, intermediate and late). In each assay a group of plants remained untreated (about two plants of each morph), a second was inoculated with TRV1 and empty TRV2 (about five plants of each morph), a third one with TRV1 and TRV2-*AqvANS* (about 13 [P] and seven [T] plants) and finally a group of plants was treated with TRV1 and TRV2-*AqvANS-NdAP3-3* (about 22 [P] and 14 [T] plants). Plants were treated by injection of 1 ml of solution with a needle syringe at the base of the stem, either at 6, 7 and 8 weeks after germination (late assay); 5, 6, 7, 9 and 10 weeks after germination (intermediate assay) or 4, 5, 7 and 9 weeks after germination (early assay).

Inflorescences were regularly inspected for signs of *ANS* or *ANS/AP3-3* silencing. For each plant five to 50 flowers were observed and for each flower several phenotypic traits were recorded: *ANS*

silencing phenotype in perianth organs, perianth organ number including the number of wild-type like, semi-transformed and transformed organs. Interesting phenotypes were photographed using an E410 Olympus camera (Japan) or using a ProGres C10 camera (Jenoptik, Germany) mounted on a SMZ1500 stereomicroscope (Nikon, Japan). Freshly sampled floral organs were also documented using a SH-1500 scanning electron microscope (Hirox, Japan). Individual organs from flowers showing different degrees of silencing as well as un-silenced controls were sampled for RNA extraction and gene expression analysis as described above.

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We want to thank Laurent Bonjean (UMR-GV, Gif s/ Yvette), Gilles Santé (IBP, Orsay) and Hervé Ferry (IJPB, Versailles) for plant care, Martine Le Guilloux (UMR-GV, Gif s/ Yvette) and Bernard Adroher (IJPB, Versailles) for technical assistance, Soizic Hupet (IJPB, Versailles) for VIGS observations, and the Plateforme de Microscopies et d'Imagerie (MNHN, Paris) where the SEM studies were conducted. This work was supported by a FRB (Fondation pour la Recherche sur la Biodiversité) grant AAS-IN-2009-040 and BG was financed by a PhD fellowship from the French MENRT.

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Figures and legends

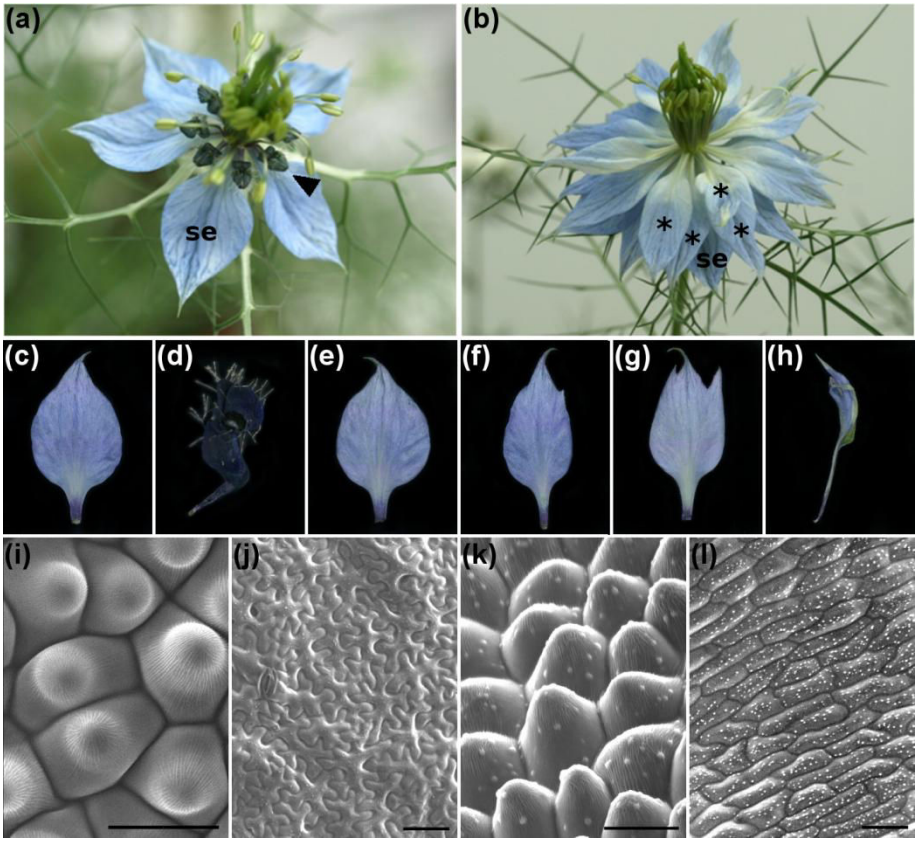

Fig.1 *Nigella damascena* flower dimorphism and perianth organ morphologies at anthesis. (a,b) Open flowers of the petalous [P] morph (a) and apetalous [T] morph. (c,d) [P] morph sepal (c) and petal (d). (e-h) [T] morph sepal (e), sepal-like organ (f), intermediate sepal-like trifid organ (g), and inner hybrid organ showing stamenoid and sepaloid characteristics (h). (i,j) Scanning electron microscopy (SEM) of the adaxial (i) and abaxial (j) surfaces of the sepal and sepal-like organs depicted in (c) and (e-h). (k,l) SEM of the petal adaxial (k) and abaxial (l) sides. se: sepals, arrowhead: petal, asterisk: sepal-like organs. Bars: (i,k) 25 µm; (j,l) 50 µm.

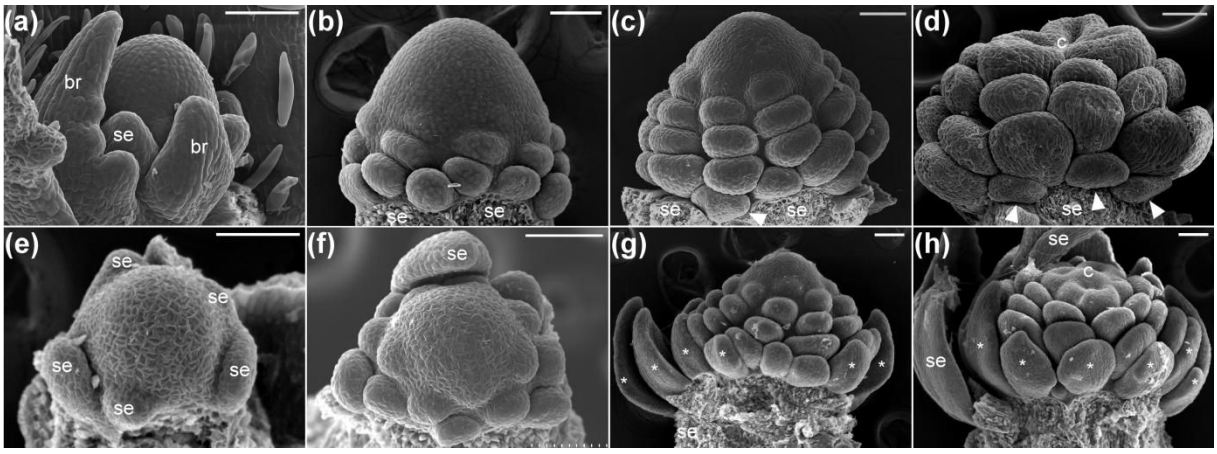

Fig. 2 SEM microphotographs of the developmental sequence of *Nigella damascena* flowers with ([P]) and without ([T]) petals. (a) [P] Floral meristem at calyx initiation – stage S-I. (b) Initiation of petal and stamen primordia – stage S-II. (c) Petal developmental delay and continued initiation of stamen primordia – stage S-III. (d) Carpel initiation, androecium differentiation and corolla development – stage S-IV. (e) [T] Floral meristem with initiated calyx – stage S-I. (f) Initiation of sepal-like and stamen primordia – stage S-II. (g) Continued sepal-like organ and stamen primordia initiation and beginning of differentiation of the outer sepal-like organ primordia – stage S-III. (h) Carpel initiation, sepal-like organ and stamen differentiation – stage S-IV. br: bracts, se: sepals, arrowhead: petal primordium, asterisk: sepal-like organ primordium, c: carpels. Bars: 100 µm.

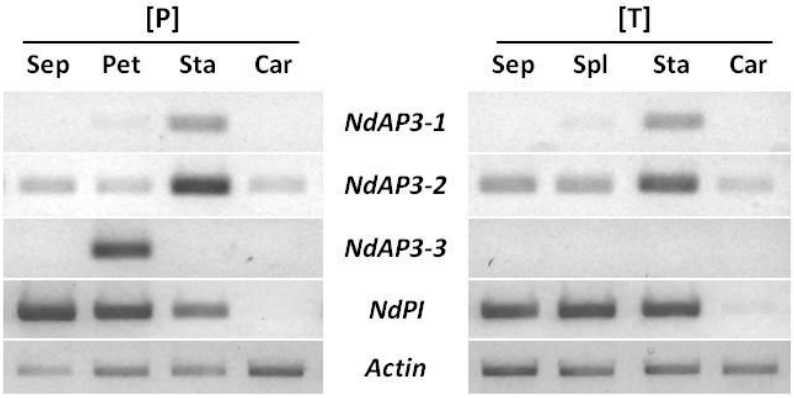

Fig. 3 *NdAP3-1*, *NdAP3-2*, *NdAP3-3* and *NdPI* locus specific RT-PCR on RNA from dissected floral organs of *N. damascena* petalous ([P]) and apetalous ([T]) flower buds (5-6 mm in diameter, stage >IV). Sep: sepals, Pet: petals, Spl: sepal-like organs, Sta: stamens and Car: carpels. Expression levels were normalized using the *ACTIN* gene.

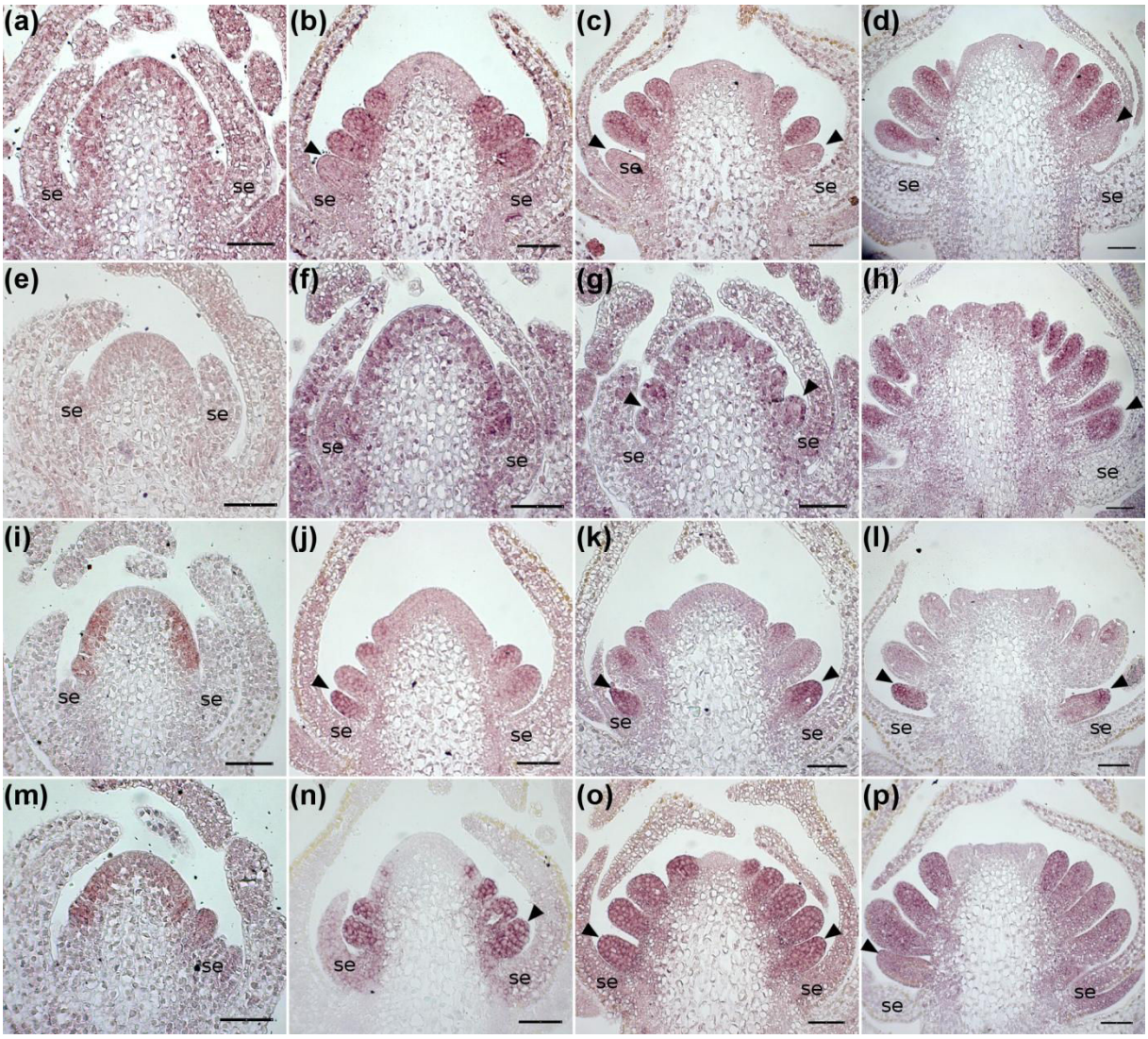

Fig. 4 *In situ* hybridization of *Nigella damascena APETALA3* and *PI* homologs in petalous, [P] morph floral meristems. (a-d) *NdAP3-1*, (e-h) *NdAP3-2*, (i-l) *NdAP3-3*, and (m-p) *NdPI*. (a,e,i) early stage S-I floral meristems with developing sepal primordia. (f,m) stage S-II meristems with undifferentiated petal or stamen primordia initiation. (b,g,I,k,n) stage S-III meristems with delayed petal primordia. (c,d,h,l,o,p) stage S-IV floral meristems with different developing organs up to the initiation of the future carpels from the flat meristem top. se: developing sepals, arrowheads: petal primordia. Bars: 100 µm.

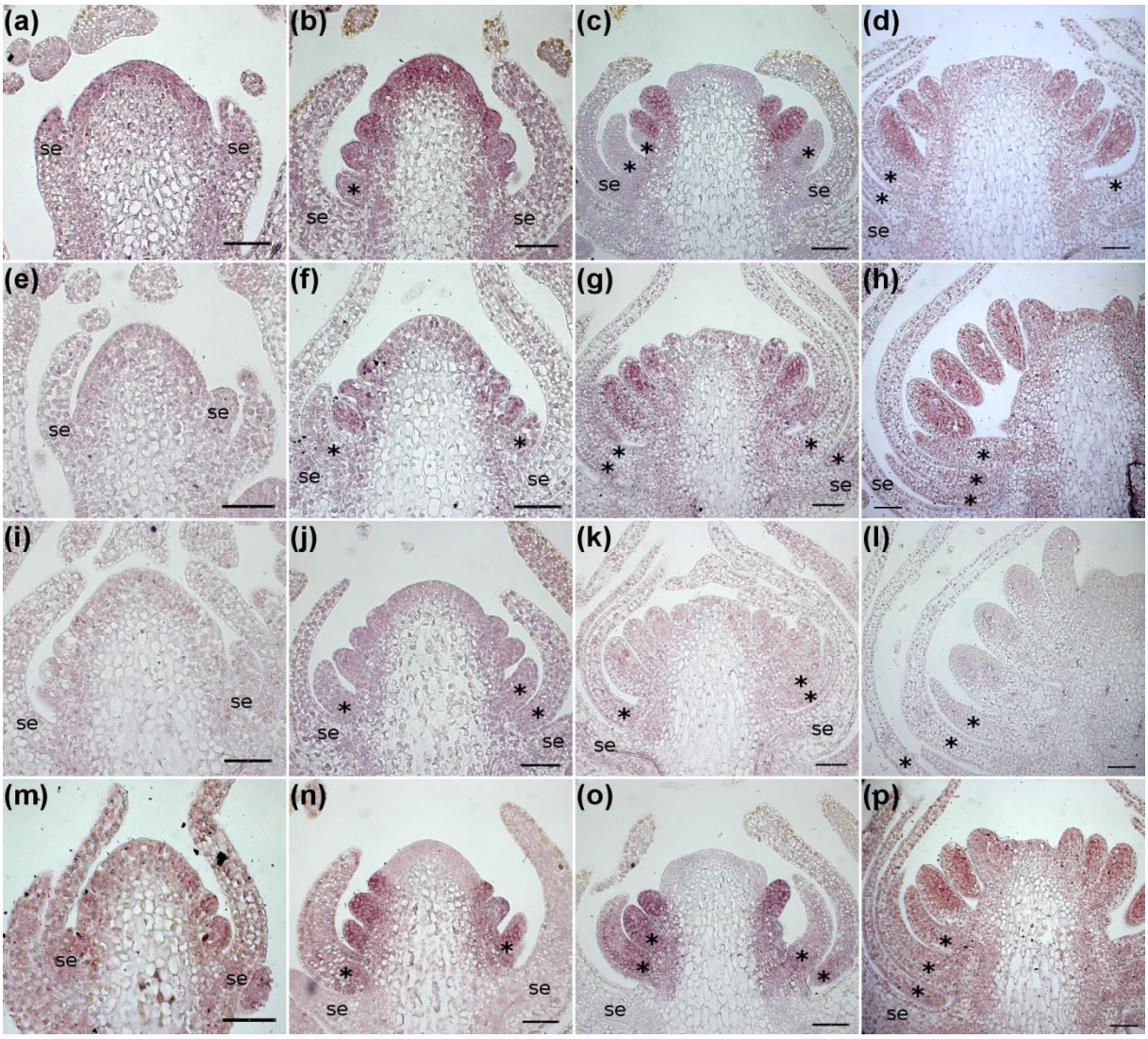

Fig. 5 *In situ* hybridization of *Nigella damascena APETALA3* and *PI* homologs in apetalous [T] morph floral meristems. (a-d) *NdAP3-1*, (e-h) *NdAP3-2*, (i-l) *NdAP3-3*, and (m-p) *NdPI*. (a,e) early stage S-I floral meristems with sepal primordia. (i,m) stage S-II meristems with few sepal-like organ and stamen undifferentiated primordia. (b-d,f,g,j,k,n,o) stage S-III floral meristems with the earliest initiated sepal-like organ primordia beginning differentiation (marked with asterisks). (h,l,p) stage S-IV floral buds at carpel initiation. The last stamen primordia can be inferred but not all intermediate primordia can be assigned to an organ type yet. se: sepals, asterisks: sepal-like organs. Bars: 100 µm.

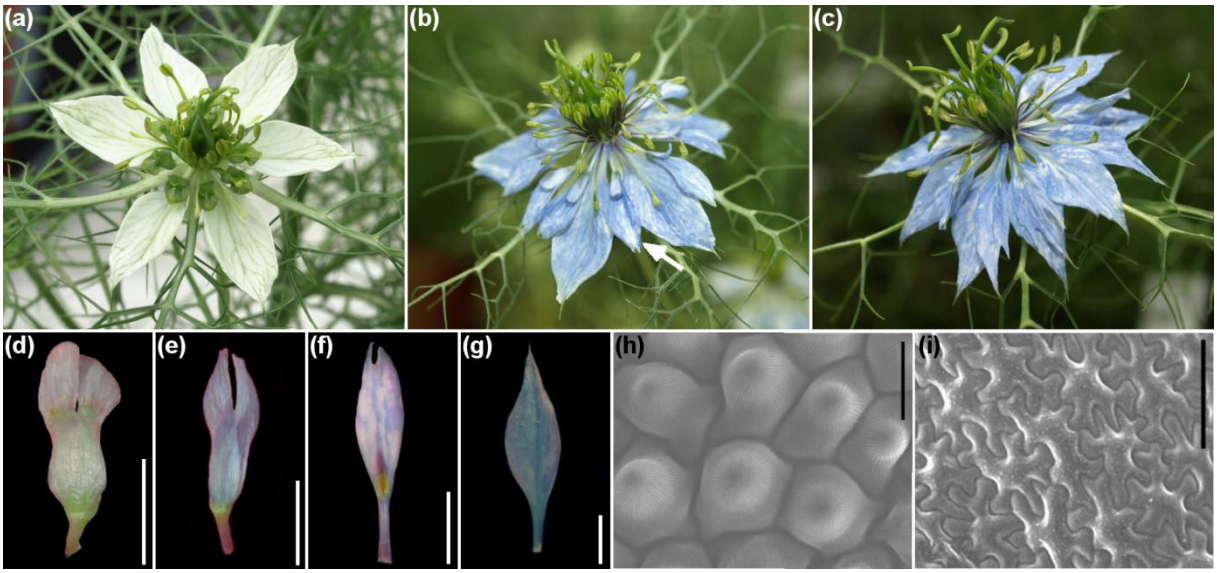

Fig. 6 Effect of *NdAP3-3* Virus Induced Gene Silencing on *N. damascena* petalous [P] plants. (a) Flower morphology under *ANS* silencing. (b-i) *ANS-NdAP3-3* silencing phenotypes. (b,c) Partially (b) and completely (c) transformed flowers. (d-g) Different degrees of petal transformation. (h,j) Adaxial (h) and abaxial (j) epidermal cellular morphology observed in the organs shown in (e-g). Arrow: semi-transformed organs. Bars: (d-g) 5 mm, (h,i) 50 µm.

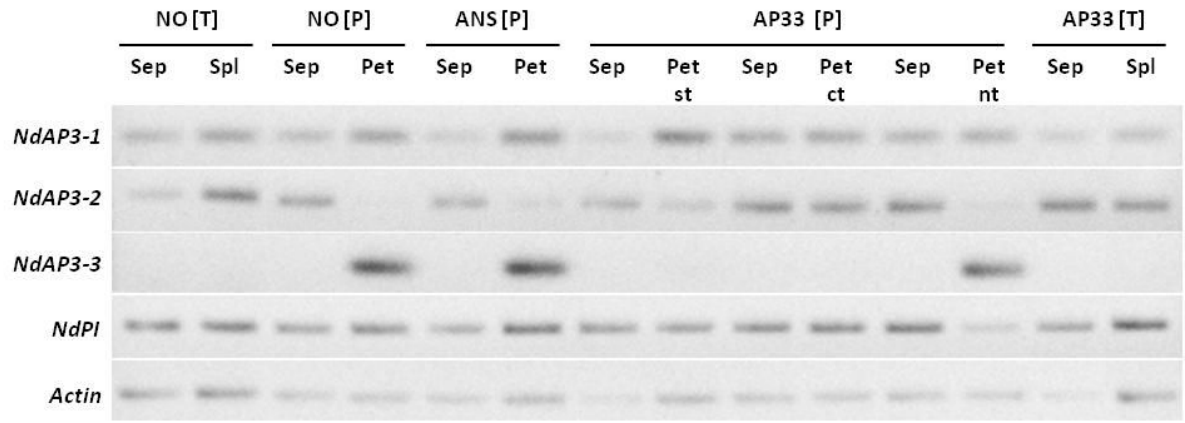

Fig. 7 *NdAP3-1*, *NdAP3-2*, *NdAP3-3* and *NdPI* locus specific RT-PCR on RNA prepared from mature perianth organs, from VIGS treated *N. damascena* plants with ([P]) and without ([T]) petals. Sepals (Sep), sepal-like organs (Spl) and petals (Pet) from untreated plants (NO), *ANS* control plants (ANS) and TRV2-*ANS-AP3-3* treated plants (AP33) showing mild silencing effects (st), strong silencing effects (ct) or no silencing effects (nt). Expression levels were normalized using the *ACTIN* gene.

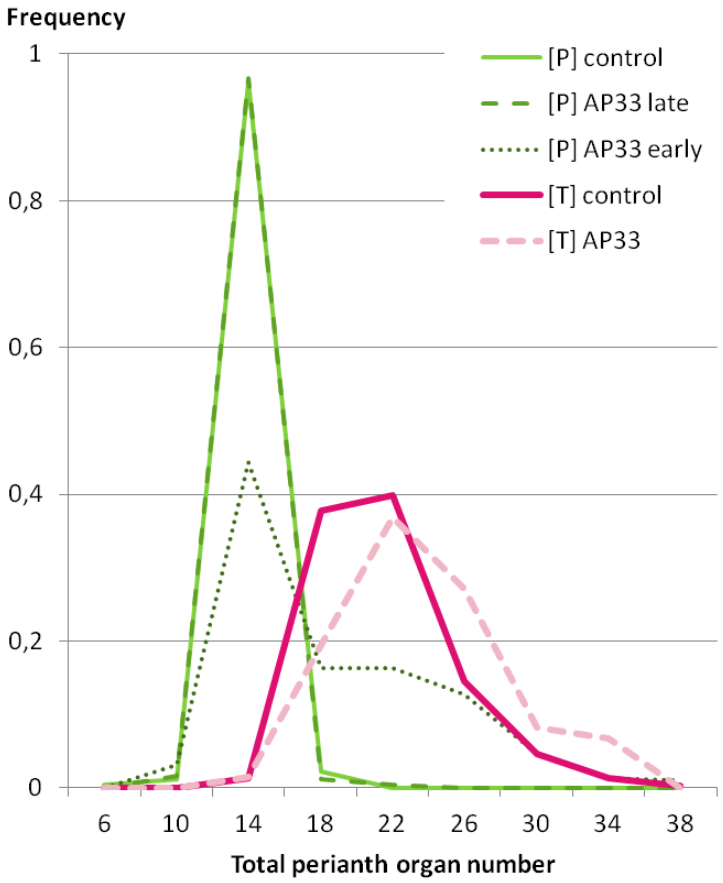

Fig. 8 Frequency distribution of perianth organ number in *N. damascena* [P] (petalous) and [T] (apetalous) plants from different VIGS treatments. Control: pooled plants from TRV2 and ANS treatments. AP33: plants treated with the TRV2-*ANS-AP3-3* construction. Early and late virus inoculation treatments are presented separately for [P] plants and pooled for [T] plants. (Number of observations: 263 [P] control, 283 [T] control, 241 [P] AP33 late, 282 [P] AP33 early, 195 [T] AP33)

Supporting information

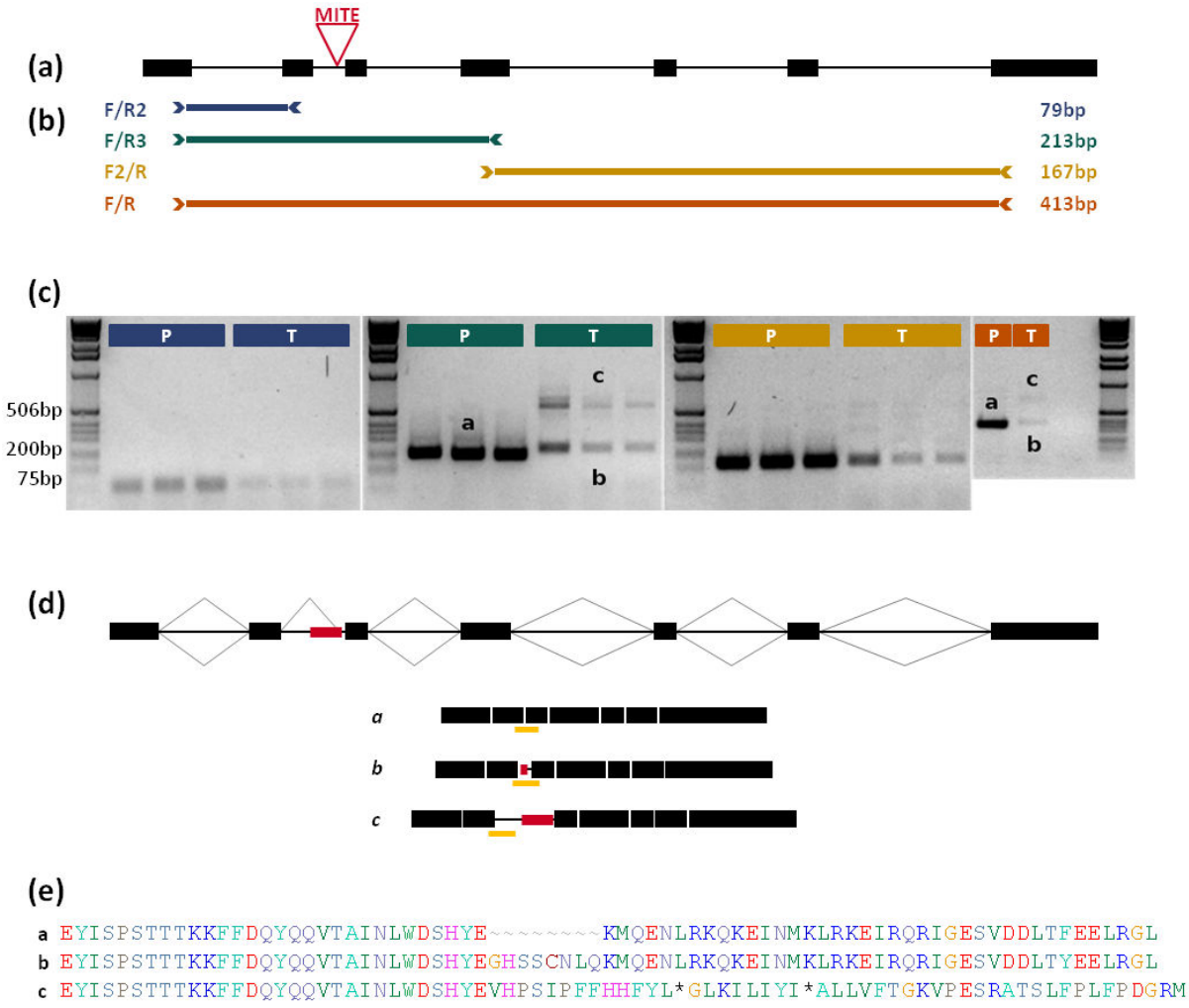

Figure S1. Alternative splicing of *Nigella damascena APETALA3-3* (*NdAP3-3*) transcripts. The presence of a MITE insertion in the second intron of the *NdAP3-3* gene is correlated with the absence of expression of a normal *NdAP3-3* transcript and the presence in very low levels of abnormally spliced transcripts. (a) Gene model with MITE insertion position (black rectangles depict exons and the lines depict introns). Floral cDNA was amplified with four different primer couples shown in (b) (primer sequences given bellow) and the separation of the respective products on agarose gel is shown in (c). These products were cloned and clones were sequenced and aligned with the previously established *wild-type* coding sequence. Based on the obtained sequences two alternatively spliced transcripts were identified and the three splicing models are depicted in (d): *a* – *wild-type* transcript. *b* and *c* – transcripts resulting from alternative splicing. The putative amino acid sequences of the

regions underlined in yellow in (d) were inferred from the sequenced clones and are shown aligned in (e). The alternatively spliced form shown in *b* contains eight extra amino acids that may interrupt a motif essential for protein function, despite not changing translation frame. In *c* a change in translation frame significantly alters the amino acid sequence and generates a premature stop codon.

Primer	Sequence 5'-3'
F	CTGAGTATATTAGTCCTTCCACCAC
F2	GGAATTGCGTGGACTTGAGCA
R	GATCCTCACCCCTGGCTTC
R2	TCCCATAGATTGATCGCAGTAACC
R3	GCTCAAGTCCACGCAATTCC

Methods S1. Characterization of candidate genes

Characterization of candidate gene coding sequences: RNA was extracted from a mix of Mornas population [P] floral buds ranging from 1 to 4 mm in diameter, using the Qiagen RNeasy kit following manufacturer instructions. DNase treatment was performed with Ambion DNaseI (Invitrogen) and single stranded cDNA was produced using SuperScriptII reverse transcriptase (Invitrogen) and random hexamers (pdN6) (Damerval *et al.*, 2007). Degenerate primers (given in supplementary table X) specific to each *AP3* paralog and *PI* were designed based on an alignment of known Ranunculaceae B-genes coding sequences (Rasmussen *et al.*, 2009). Each gene was amplified by two rounds of nested or semi-nested PCR. Single bands of expected size were sequenced directly and sequence identity was confirmed by BLAST**.**

Characterization of full length mRNAs: RACE PCR was performed on total RNA extracted from a mix of Mornas [P] floral buds having less than 2.5 mm in diameter, using Trizol (Invitrogen). Approximately 2.5 µg of total RNA were processed through dephosphorylation, mRNA cap removal and RNA oligo ligation, using the GeneRacer kit as per manufacturer instructions (Invitrogen). The final RNA was transcribed using SuperScript III reverse transcriptase. 5' and 3' RACE were performed for each locus using two nested gene specific primers (given in supplementary table X) along with the supplied 5', 5'-Nested, 3' and 3'-Nested primers. Products from nested PCRs were

purified and cloned using TOPO-TA plasmid vector (Invitrogen). For each reaction 12 to 20 clones were screened and between 5 and 10 were sequenced. Resulting 5' and 3' sequences were aligned against previously known cDNA sequences and full length consensus sequences were generated.

Characterization of *NdAP3-3* genomic locus: Genomic DNA was prepared from 4 [P] and 4 [T] individual plants from four different origins (Mornas, Royal Fleur, Truffaut and Vilmorin) using DNeasy Plant Mini kit (Qiagen). Gene specific forward and reverse primers (Table S2) were designed to fit regions predicted to fall within exons 1 and 7 respectively, based on an alignment of *N. damascena* coding sequences and related species *Aquilegia coerulea* genomic sequence for the homologous locus. PCR products were sequenced and the obtained sequences were aligned and their identity verified by aligning against previously known CDS.

Table S1. Segregation analysis of floral morph, P locus genotype and *NdAP3-3* genotype. 97 petalous plants ([P]) and 39 apetalous plants ([T]) were studied. P locus genotype, responsible for floral Morph, was determined based on test-cross results. *NdAP3-3* locus was genotyped as to the absence (-/-) or presence of MITE insertion, in one (-/+) or two (+/+) alleles.

		***NdAP3.3* genotype**		
Morph	**P locus genotype**	- / -	- / +	+ / +
[P]	*PP*	20	0	0
	Pp	0	77	0
[T]	*pp*	0	0	39

Table S2. List of primers used in this work.

Purpose	Gene	Forward	Reverse
B genes amplification, degenerate primers	*AP3.1*	AP31 Fwd ext GCYVRVGARCTHAVYGTTC	AP31 Rv GGHWGRYTWGGHTGYARRCG
		AP31 Fwd int GCYSARGTTKCTYTHATYATG	AP31 Rv GGHWGRYTWGGHTGYARRCG
	AP3.2	AP32 Fwd ext CTYWSYGTTCTYTGYGAYGCYCAAG	AP32 Rv ext GCDAGRSKYAAWCCATADGWRCC
		AP32 Fwd int WWCKGRDRTVRATCTBTGG	AP32 Rv int RYCMTGWARRYTKGGYTG
	AP3.3	AP33 Fwd ext GYGATGCYGARGTBTCKCTYRTC	AP33 Rv CRTAATCVCCTTCRWAGTAAG
		AP33 Fwd int TYRTCATGTTYTCYWGACYGG	AP33 Rv CRTAATCVCCTTCRWAGTAAG
	PI	PI Fwd ext GAAAGCTARRGAGATWRCTGTTC	PI Rv AAGGCATSTVGRAGGATARTC
		PI Fwd int CTARYACTRRCAAGRTGWHKGAG	PI Rv AAGGCATSTVGRAGGATARTC
NdAP3-3 5' and 3' RACE gene specific primers	*AP3.3*		gAP33R GATCCTCACCCCTGGCTTC gAP33R2 TCCCATAGATTGATCGCAGTAACC
		gAP33F CTGAGTATATTAGTCCTTCCACCAC gAP33F2 GGAATTGCGTGGACTTGAGCA	
NdAP3-3 genomic locus amplification	*AP3.3*	gAP33F CTGAGTATATTAGTCCTTCCACCAC	gAP33R GATCCTCACCCCTGGCTTC
Expression analysis – RT-PCR	*AP3.1*	AP31 U CCTAACACCACAATGAAACT	AP31 L CAATCCTCAATGAGCTATCT
	AP3.2	AP32 U GATGACCTTACCTTCCACCAA	AP32 L TGCGAGAGTAATCGTAGACTG
	AP3.3	AP33 U ATCAACAGGTTACTGCGATCA	AP33 L GATACTTCCGATCACGAACAA
	PI	PI U GTCCTAACTCCACGCTGATAA	PI L CCTTCGATGTCCATTTGCT
	Actin	Act U AACTGGGATGATATGGAGAA	Act L CCTCCAATCCAGACACTGTA
Expression analysis - *In situ* hybridization	*AP3.1*	iAP31 F3 CCACCTTACCTAACTTTTCC	iAP31 R4 T7 TGTAATACGACTCACTATAGGGC TCAGTTCAGATATTTAACC
	AP3.2	iAP32 F3 ACCCCTACCCCTCTTCTT	iAP32 R3 T7 TGTAATACGACTCACTATAGGGC AGGCTTAAACCATAAGAGCC
	AP3.3	iAP33 F4 CTTTACCTGGAGTATCATGG	iAP33 R2 T7 TGTAATACGACTCACTATAGGGC CTGAGACCTAATGTATTCTCC
	PI	iPI F3 GGAAAGATTGAGATCAAAAG	iPI R4 T7 TGTAATACGACTCACTATAGGGC TGCTCATTACTTGTATCACC

Concluding remarks

During the preparation of this article, a parallel study on the evolutionary origin of apetalous flowers within the Ranunculaceae family was published, encompassing a description of the *Nigella damascena* apetalous mutant and similar expression and segregation studies of the *AP3-3* homolog, including a description of a mutant allele containing the same transposable element insertion as described in our work (Zhang *et al.*, 2013). Despite sharing a common thread of results, the two works diverge significantly in the interpretation of two aspects of those results, the first being about the relationship of the transposable element insertion with the apetalous mutant phenotype, and the second pertaining to the scope of the *AP3-3* role in the *Nigella damascena* flower development and its implications.

We believe that the authors were too hasty to interpret the close association of the MITE-bearing allele with the mutant phenotype, demonstrated by co-segregation analysis, as evidence for causality, when in fact at that point, it merely demonstrates correlation. "This […] confirmed that loss of petals in the *Nigella* mutant was indeed caused by the MITE insertion" (Zhang *et al.*, 2013). There are two steps in the line of thought that links the MITE insertion to the mutant phenotype, neither of which we believe can be fully confirmed by the results of co-segregation alone. a) MITE insertion causes *NdAP3-3* inactivation. b) *NdAP3-3* inactivation causes phenotype. Hence, c) MITE insertion causes phenotype. While our study provided solid substantiation for the second premise, the first part of that syllogism remains speculative. Support for the disruptive effect of MITE insertion on *NdAP3-3* expression in that article was taken from the literature. Indeed transposable elements (TEs) have been shown to have an important mutagenic effect via insertion within coding sequences (leading to the production of wrong or truncated transcripts), and non-coding sequences such as introns (which can affect splicing leading to the production of alternative or truncated transcripts) and regulatory regions (which in the case of DNA transposons can lead to modifications of transcription activity, initiation or termination via the transposable elements own regulatory promoters and terminators) (reviewed in Feschotte & Pritham, 2007). However, upon close inspection of cited literature and other works, we found that evidence for the impact of MITE insertion on absence of expression is circumstantial and contradictory. Growing evidence for the special relationship between MITEs and plant genes has for

some time now prompted speculation over the potential of MITE activity in the evolution of gene regulation and genome diversification (Bureau *et al.*, 1996; El Amrani *et al.*, 2002; Santiago *et al.*, 2002; Oki *et al.*, 2008; Benjak *et al.*, 2009). However, the association between MITE elements and genes is only an indication of their potential role as a generator of variability, and some authors have been cautious to point out that so far no, or few, molecular experimental evidence unambiguously confirms such effects of MITE insertion (Casacuberta & Santiago, 2003; Feschotte, 2008). Indeed, reports on MITE insertion effect on gene expression have produced ambiguous results. Some reports, such as the study of one of the spontaneous *waxy* mutants in maize (Bureau & Wessler, 1992), or of the rice *slender glume* mutant phenotype (Nakazaki *et al.*, 2003), have shown an association between mutant phenotypes and the insertion of MITE elements either in the coding sequence or intronic regions without direct molecular evidence for the impact of MITE insertion on gene regulation. A study of the *mPing* element in rice concludes that insertion of *mPing* MITE has a neutral or minimal effect on gene transcription, as only two out of the eight studied insertions are associated with a decrease in gene expression levels and none affected splicing (Naito *et al.*, 2006), while a more recent study revealed significant alteration of gene expression by *mPing* insertion only under stress conditions (Yasuda *et al.*, 2013). In another study, Lu et al. (2012) showed that genes with MITE insertions upstream or downstream and within introns show significantly lower expression levels than genes with no association with MITEs. However, upon individual inspection of genes displaying MITE insertions, no significant differences of expression levels were found between the alleles with and without MITE insertion (Lu *et al.*, 2012). The insertion of the *Kiddo* element in the promoter of the *rice ubiquitin2* gene was shown to be associated with two contradictory effects. On one hand its presence increases *in vitro* transcription rates, and on the other hand, it induces epigenetic modifications that lead to transcriptional silencing (Yang *et al.*, 2005). It is clear that this is a field in development, and that further studies are needed in order to confirm both general and particular trends of MITE insertion impact on gene regulation and coding capacity. During our research we attempted to determine the impact of the MITE insertion on the expression of *NdAP3-3* via disruption of splicing mechanisms. While our results suggest that MITE presence does have an impact on the splicing pattern, we carefully point out that they cannot alone explain the absence of expression in the mutant

form. Therefore additional studies on the possible mechanisms of gene silencing in relation to but not exclusively based on MITE insertion are underway at our lab. Besides the potential direct mutagenic effects of transposable element insertion, TEs may influence gene expression by transferring to neighboring sequences the epigenetic marks that are targeted at their own silencing as part of the plant's defense mechanism (Weil & Martienssen, 2008). Repression of TE activity involves both post-transcriptional epigenetic mechanisms, such as RNAi mediated silencing, and transcriptional repression mechanisms, such as chromatin modifications, including histone modification, DNA methylation and chromatin packaging that can also be RNAi-mediated (Slotkin & Martienssen, 2007; Rigal & Mathieu, 2011). Despite being non-autonomous, therefore incapable of moving on their own, MITEs represent one of the most abundant transposable elements in plant genomes suggesting that they somehow avoid repression by host genomes (Casacuberta & Santiago, 2003). However, although the fact that MITEs are not transcribed means that they cannot be inactivated by transcriptional and post-transcriptional repression mechanisms, silencing associated processes such as DNA methylation have been shown to influence MITE activity (Ngezahayo *et al.*, 2009). Indeed, in wheat, a large proportion of sRNAs matching transposable elements have been show to correspond to MITEs suggesting that they are the target of epigenetic silencing mechanisms (Cantu *et al.*, 2010). Therefore efforts to understand the potential connection between MITE insertion and *NdAP3-3* silencing will be focused on the study of DNA methylation and histone tailing patterns. Additionally we do not discard the hypothesis that MITE insertion is unrelated with the event that caused gene silencing. Indeed, the absence of expression of *NdAP3-3* could be caused by a mutation at the promoter level or within other cis-regulatory sequences. The primary inactivation of the gene by such a mutation could then be followed by accumulation of further mutations such as the MITE insertion. Alternatively, absence of *NdAP3-3* expression could originate at a molecular level via mutation of an upstream trans-regulator of its activity. Potential candidates for *AP3-3* regulators could be taken from the *Arabidopsis* model where *AP3* expression is activated by co-factors *UFO* and *LFY* (see Introduction). However, because the *NdAP3-3* gene co-segregated perfectly with the locus responsible for the mutant phenotype, this hypothesis is dependent on a close physical proximity between both loci.

The second divergence between ours and Zhang *et al.*'s (2013) work has to do with the role of *NdAP3-3* in floral development and the extent of its conservation within the Ranunculaceae family. Zhang *et al.*'s work follows on the footsteps of previous works such as Rasmussen and colleagues (2009), by adding supporting evidence to the idea of a conserved petal identity program in this family. Indeed, before Rasmussen *et al.*'s work, the prevailing view based on morphological observations suggested that petals had originated several times independently within basal eudicots with convergent recruitment of similar molecular tools (Hoot, 1991). Rasmussen *et al.*'s findings of a petal-specific expression of *AP3-3* homologs across the petalous member of the Ranunculaceae family (and a respective absence from apetalous species) suggested a single origin for petals in the Ranunculaceae and Berberidaceae families, and a commonly inherited petal identity program rather than different episodes of convergent evolution. A single origin for petals implied that independent processes were at the origin of the different petal-loss events within the family, a hypothesis which gained solid support from Zhang *et al*. Furthermore, in addition to Rasmussen *et al.*'s evidence for conservation of petal-identity programs between Ranunculaceae and the sister clade Berberidaceae, Hu *et al*. (2012) found support for the conservation of petals in the lower family Lardizabalaceae, suggesting an ancestral origin for petals within the Ranunculales that predates the divergence of the Lardizabaleceae, despite their morphological diversity. Prior to our work, functional evidence supporting the involvement of *AP3-3* homologs in the specification of petal identity in this family was only available for the *Aquilegia*, in which *AqAP3-3* silencing leads to a transformation of petals into sepals (Sharma *et al.*, 2011). In our work, we found that the inactivation of *NdAP3-3* (whether in the mutant form or by artificial silencing) leads not only to a transformation of organ identity but also an increase in perianth organ number and a disruption of the perianth-stamens boundary. While both works support the conserved role of *AP3-3* in petal-identity programs within the Ranunculaceae family, it is unclear whether the role in organ number and organ boundary regulation is also conserved as there are no other reports that we know of showing such a complex role for the *AP3-3* gene. As briefly mentioned in the discussion of our article, because the different effects of *AP3-3* silencing in the two species coincides with two different phyllotaxis, we hypothesize that the additional role of *AP3-3* in the regulation of the perianth-stamens boundary is tied with the spatial organization of the two primordia

within the meristem. That is, that the spiral phyllotaxy of the *Nigella damascena* provides a looser base for the reorganization of organ identities after petal-loss giving rise to a hybrid zone with mixed identities, whereas the *Aquilegia* whorled phyllotaxy implicates a much stricter separation of organs that is conserved after the loss of petals. Phyllotaxis is remarkably variable across the Ranunculaceae, with many transitions between spiraled and whorled phyllotaxy, including states of irregular patterning both at the genus and species level. Additionally mixed patterns can often be observed within meristems both in a spatial and temporal way (Tucker & Hodges, 2005; Ren *et al.*, 2009, 2010, 2011; Zhao *et al.*, 2011). This lability of floral phyllotaxis is accompanied by an equally variable range of perianth architectures and organ identities. Therefore, it would be interesting to fit a comparative morphological study of perianth composition, phyllotaxis and organ number within an evolutionary context. Additionally, similar functional studies to the ones performed in *Aquilegia* and *Nigella* are necessary to understand the extent of conservation of the *AP3-3* role in floral development.

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Chapter 2. Ecological and evolutionary significance of the *Nigella damascena* floral dimorphism

"Where ignorance lurks, so too do the frontiers of discovery and imagination."

— Neil deGrasse Tyson, astrophysicist

Résumé en français du deuxième chapitre

Signification écologique et évolutive du dimorphisme floral chez *Nigella damascena*

Le deuxième aspect de cette thèse concerne l'étude de la signification évolutive du polymorphisme floral observé chez *Nigella damascena,* dans le but d'évaluer le potentiel évolutif des deux morphes floraux dans des conditions naturelles. Une prospection antérieure de populations naturelles de *Nigella damascena* dans le sud de la France avait révélé une prédominance des populations monomorphes composées de type sauvage [P], avec pétales. Les rares cas de populations polymorphes sont souvent associés à des territoires urbains où le morphe [T] est plus fréquent, cultivé à la fois dans des jardins, prairies fleuries et des champs laissés en jachère. Le manque de populations polymorphes naturelles facilement accessibles a conduit à la création d'une population expérimentale semi-naturelle sur laquelle effectuer les mesures et observations. Des observations *in situ* ont été réalisées au cours des saisons de floraison des deux premières générations. Des données de production de semences ont été recueillies sur un échantillon de capsules de la première génération. Un échantillon de descendants de la première génération a fait l'objet d'observations en serres par Mathilde Latron (étudiante en M2). L'ensemble de ces données ont été utilisées pour analyser le potentiel évolutif des morphes en populations naturelles mixtes. Tout d'abord nous avons cherché à élucider les modes possibles de reproduction de chaque morphe via la caractérisation *in situ* des pollinisateurs de *Nigella damascena* et de leur comportement vis à vis des deux formes. En parallèle nous avons effectué une description quantitative de caractères végétatifs et reproducteurs des deux morphes dans des conditions naturelles, qui a été utilisée pour calculer des mesures de vigueur, morphologie florale et succès de reproduction. Des traits associés au succès reproducteur ont été utilisés pour estimer un ensemble de composants de valeur sélective qui ont été utilisés pour détecter des gradients de sélection sur des traits floraux. Enfin, la combinaison de ces données avec des observations sur l'évolution du morphe-ratio entre les deux générations a permis de formuler des hypothèses sur l'évolution du polymorphisme dans les populations naturelles, et à inférer les conséquences de l'hybridation récurrente entre les populations naturelles composées exclusivement du morphe [P] et des plantes de morphe [T] provenant de populations artificielles. Nous avons observé un effet qualitatif du morphe

floral sur le comportement des pollinisateurs, qui a un impact potentiel sur le mode de reproduction. La forme [T] est peu visitée par des pollinisateurs et semble se reproduire majoritairement par autofécondation, tandis que la forme [P] avec pétales est plus fréquemment visité et potentiellement allogame. De manière surprenante, nos résultats suggèrent un avantage de la forme sans pétales [T] par rapport au type sauvage [P]. L'origine de cet avantage pourrait résider dans le déséquilibre de liaison entre le locus P et un gène de vigueur, provenant de la structure génétique étroite de notre matériel végétal, dont les limites sont discutées. Nous avons trouvé aussi une dépression de consanguinité dans la descendance des plantes [T], en lien avec leur mode de reproduction majoritairement par autofécondation. Ainsi, la compréhension de l'évolution des populations polymorphes nécessite une appréciation de l'équilibre entre ces deux facteurs. Nos observations suggèrent que la dépression de consanguinité associée au mode reproduction du morphe [T] peut compenser dans une certaine mesure l'avantage lié à sa génétique et mener le morphe-ratio vers un scénario plus équilibré. Cependant, au cours de cette étude, seulement deux générations ont été observées, ce qui est limitant pour nos hypothèses sur le résultat d'une situation polymorphe. En conséquence, la poursuite sur plusieurs générations de l'étude de l'évolution du morphe-ratio, des modes de reproduction et de la valeur sélective des deux morphes est nécessaire pour discerner des tendances évolutives plus solides dans les populations naturelles polymorphes de *Nigella damascena*.

Preamble

The second aspect of the present thesis concerned the study of the evolutionary significance of the *Nigella damascena* floral polymorphism, in an attempt to decipher the evolutionary potential of the two floral morphs in natural populations. A previous survey of natural *Nigella damascena* occurrences in French territories revealed a predominance of monomorphic populations composed exclusively of the petalous wild-type [P]. The rare instances of polymorphic populations are frequently associated with urban territories where the morph [T] is more frequent, being cultivated both in home gardens, flowery meadows and fields left fallow. The lack of readily accessible natural polymorphic populations led us to create of a semi-natural experimental population on which to carry observations. *In situ* observations were made during the flowering season of the first two generations. Seed production data was collected on fruits sampled in the first generation. A sample of first generation descendants was observed in greenhouse by Mathilde Latron (second year master student). The sum of these observations was used in a four way approach to understand the evolutionary potential of natural populations in polymorphic situations. Firstly we aimed at elucidating the possible modes of reproduction of each morph via *in situ* characterization of pollinator assemblage and pollinator behavior in relation to both morphs. In parallel we performed a comparative description of the two morphs vegetative and reproductive aspects in natural conditions which was used to derive measures of vigor, floral morphology and reproductive success. Traits associated with reproductive success were used to estimate a set of fitness components which were used to detect potential selection gradients on individual floral traits. Finally, the integration of these studies with the observations of morph-ratio evolution between the two generations should allow us to formulate hypotheses on the evolution of the *Nigella damascena* polymorphism in natural polymorphic populations, and to understand the consequences of recurrent hybridization between natural populations composed exclusively of [P] morph and [T] morph plants from artificial populations. This chapter presents the rationale and the results of those observations organized into Introduction, Material and methods, Results and Discussion.

Introduction

Flowers displays a remarkable variability of architectures across angiosperms, particularly at the perianth level, much of which stems from changes in developmental processes, which are often of homeotic nature (Theißen, 2010). However, understanding the genetic and developmental origin of diversity alone does not lead to a complete understanding of the evolutionary history of such diversity. The flower, being the plant's reproductive unit, plays a pivotal role in its fitness, and plants, being sessile organisms depend more than others on external factors for the success of reproduction, and in particular of mating with another plant (Stebbins, 1970; Barrett, 2010). Among those factors, the biotic vectors that transport pollen between flowers are likely to produce the strongest interactions with floral traits (Barrett, 2010). Therefore, understanding of the relationships between flowers and pollinating animals is fundamental in solving the history and mechanisms of floral architecture diversity. The study of the ecological aspects of diverging floral phenotypes within the same species has been proposed as an ideal tool for exploring the evolutionary significance of flower architecture diversity (Hintz *et al.*, 2006). The *Nigella damascena* floral dimorphism, comprising a transition from a petalous to an apetalous form, presents the perfect opportunity to carry such a study. Flowers of the wild-type, or [P] morph, have a differentiated perianth with five conspicuous sepals and eight nectariferous petals, while the mutant [T] morph produces flowers with no nectariferous petals but a greater number of sepal-like organs (for a more detailed description of the *Nigella damascena* flower morphology and floral dimorphism see introduction and Chapter 1). The *Nigella damascena* has a Mediterranean area of distribution and an annual life cycle with flowering occurring in early spring. After germination in late summer, vegetative growth occurs under the form of a leafy rosette between autumn and spring when floral transition produces one or more floral stems with flowers arranged in panicles. While the petalous wild-type [P] morph can be found throughout the species natural area of distribution, the mutant form [T] has a more limited distribution being more frequently found in urban environments where it is cultivated as an ornamental plant. Nevertheless, circumstantial evidence for the existence of natural polymorphic populations was revealed to us during a previous study of floral morphology (plant material described in Chapter 1). Additionally, reports of mixed populations

outside the natural range of the species distribution can be found in the literature (Boufford, 1997). The ephemeral nature of such populations, particularly those found outside the original area of distribution of the species, suggests that their maintenance is strongly dependent on environmental conditions. One hypothesis for the recurrent formation of such populations is the occasional release of the [T] morph from artificial cultivation into the wild and into natural [P] morph populations. Whether this occurs via pollen transportation by insects or by seed transportation by animals or man could be an interesting subject of research.

In the interest of understanding the role of natural homeotic mutants as vehicles of evolutionary novelty, and distinguishing between gradual and saltational evolutionary modes, the genetic bases of such mutant forms must be dissected (Theißen, 2009, 2010). The genetic mechanisms underlying the *Nigella damascena* floral dimorphism were investigated in the previous chapter. The mutant apetalous form was confirmed to be specified by a recessive allele in a single locus which we identified as the *APETALA3-3* gene. In this chapter the same generic notation will be adopted for the responsible locus, the *P* locus, and its alleles, *P* and *p*. These can produce the homozygous *PP* and heterozygous *Pp* combinations, which due to the dominant nature of the *P* allele responsible for the petalous phenotype produce the morph [P], or the recessive homozygous *pp* combination that produces the apetalous phenotype [T] morph (**Figure 1**).

Figure 1. The *Nigella damascena* floral dimorphism, the two floral phenotypes and corresponding genotypes at the *P* locus level. White arrowhead: petals, sep: sepals, spl: sepal-like organs.

As previously pointed out, in order to understand the potential of homeotic mutants as roots for new evolutionary lineages, one must study the ecological and population dynamics mechanisms underlying their maintenance in natural conditions (Hintz *et al.*, 2006). Therefore, we set out to study the two *Nigella damascena* floral morphs in an experimental polymorphic population, with a particular concern for the impact of the dimorphism on pollinator behavior, reproductive success and overall fitness, and floral trait evolution. As briefly mentioned in the general introduction, *Nigella damascena* flowers, much like the flowers of the remaining members of the *Nigella* genus, display a set of morphological traits that are likely adaptations to an out-crossing reproduction mode taking advantage of insects, the most notable of which is the presence of nectariferous petals (Zohary, 1983). Additionally, anthesis is marked by a sequence of intricate style and stamen movements, which have been interpreted as an adaptive mechanism to ensure contact between pollen loaded pollinators and the style crest surface as the styles curve downward and twist in a corkscrew fashion (Toxopéus, 1927; Zhao *et al.*, 2011). However, *Nigella damascena* flowers have no issues of self-incompatibility (Greyson & Sawhney, 1972; Raman & Greyson, 1977), and self-pollination is supposed to occur in the absence of pollinators (Toxopéus, 1927). Additionally, successful selfing has been achieved in greenhouse assays performed in our lab. The *Nigella damascena* floral dimorphism affects some of the traits believed to contribute to pollinator attraction and pollination efficiency, such as the presence of petals and nectar, and the number of showy organs. Therefore, we were particularly interested in studying the impact of those changes in insect behavior, and what effect insect behavior has on reproduction mode. Namely, if insects visit preferentially the morph with petals, what will be the relative importance of cross-pollination and selfing in each morph, and what will be their contribution to both morphs reproductive success. Absolute fitness of a plant cannot be measured directly, as it may depend on many random factors, but can be estimated through its partial components, that is traits, or combinations of traits believed to be associated with it or to contribute to it, such as survival and mating success (Brodie III *et al.*, 1995). In hermaphroditic plants, overall reproductive fitness should be estimated both in terms of the female and male functions, i.e. seed production and seed paternity, respectively (Barrett, 2010). However, while seed production can be easily evaluated without any particular device, paternity assignment requires extensive molecular tools and the development of

genetic markers which may not be readily available for all species of interest. Therefore, despite being incomplete and representing only 'one half' of the equation, the most common approach when estimating fitness is to equate it to the relative seed production and quality (Gómez *et al.*, 2006; Ziermann *et al.*, 2009; Parachnowitsch & Kessler, 2010; Sletvold *et al.*, 2010). Finally, we were also interested in assessing the ecological aspects of floral trait evolution relative to both morphs. If pollinator behavior or visitation patterns do indeed shift in relation to floral morph in a way that involves different interactions between pollinators and flowers, we might expect those differential interactions to exert diverging selective pressures on floral morphology and individual floral traits in both morphs. Morph-specific selection on floral traits can occur, for example, if as a result of floral morphology shifts, pollinator attraction and efficiency depends on different floral traits in each morph.

Material and methods

Plant material

Plants used in the production of a polymorphic population originated from two natural populations, the polymorphic Mornas population (Vaucluse, South of France, accession 04-98 of the French National Museum of Natural History) and the monomorphic [P] population Araguina (Bonifacio, Corsica, Conservatoire Botanique National Méditerranéen de Porquerolles), and three cultivated commercial seed lots which are mostly monomorphic [T] (Royal Fleur, Truffaut and Vilmorin).

Production of a polymorphic population

A polymorphic population containing plants with and without petals ([P] and [T] floral morphs respectively) was obtained in a series of test crosses between [P] and [T] plants (**Figure 1**). In the first cross, flowers from two homozygous [P] plants originating from the natural population Araguina were pollinated with recessive [T] pollen from plants originating from commercial seed lots (Royal Fleur for Araguina-1 and, Vilmorin and Truffaut for Araguina-2a and Araguina-2b, respectively). This produced three families of 17 to 20 heterozygous [P] plants each. For each plant six flowers were selected and emasculated (removal of anthers) to be test-crossed with recessive pollen from [T] plants of commercial seed lots (Royal Fleur, Truffaut and Vilmorin) and the natural population of Mornas. These crosses produced 336 capsules, each containing a mixed progeny of 50% heterozygous [P] plants and 50% homozygous [T] plants. Each capsule was equally sampled (4 seeds per capsule x three lots) to obtain three equivalent lots of approximately 1300 seeds each (**Figure 2**).

The experimental set-up

Three field plots of $100m^2$ were minimally prepared to receive three population replicates. One replicate is situated at Gif-sur-Yvette (North of France) in the UMR-GV site (MLN) and the other two in the Montpellier region (South of France), one in the CNRS-CEFE campus (CEF) and the other at the INRA-Mauguio station (MAG). Two of the seed lots were sown by broadcasting the seeds in the

field at the CEF and MAG sites in the autumn of 2011, whereas a third lot was sown in greenhouse in the beginning of spring 2012 and transferred to the MLN field plot after one month. All three fields were minimally maintained and, apart from a sample of plants selected for observations and seed collection, plants were left untouched to reproduce and sow freely. Generations G_0 and G_1 (**Figure 3**) refer to plants that flowered in the field plots in 2012 and 2013, respectively. Plants derived from the seeds collected on G_0 plants (mothers) and grown in greenhouse are referred to as the "progeny".

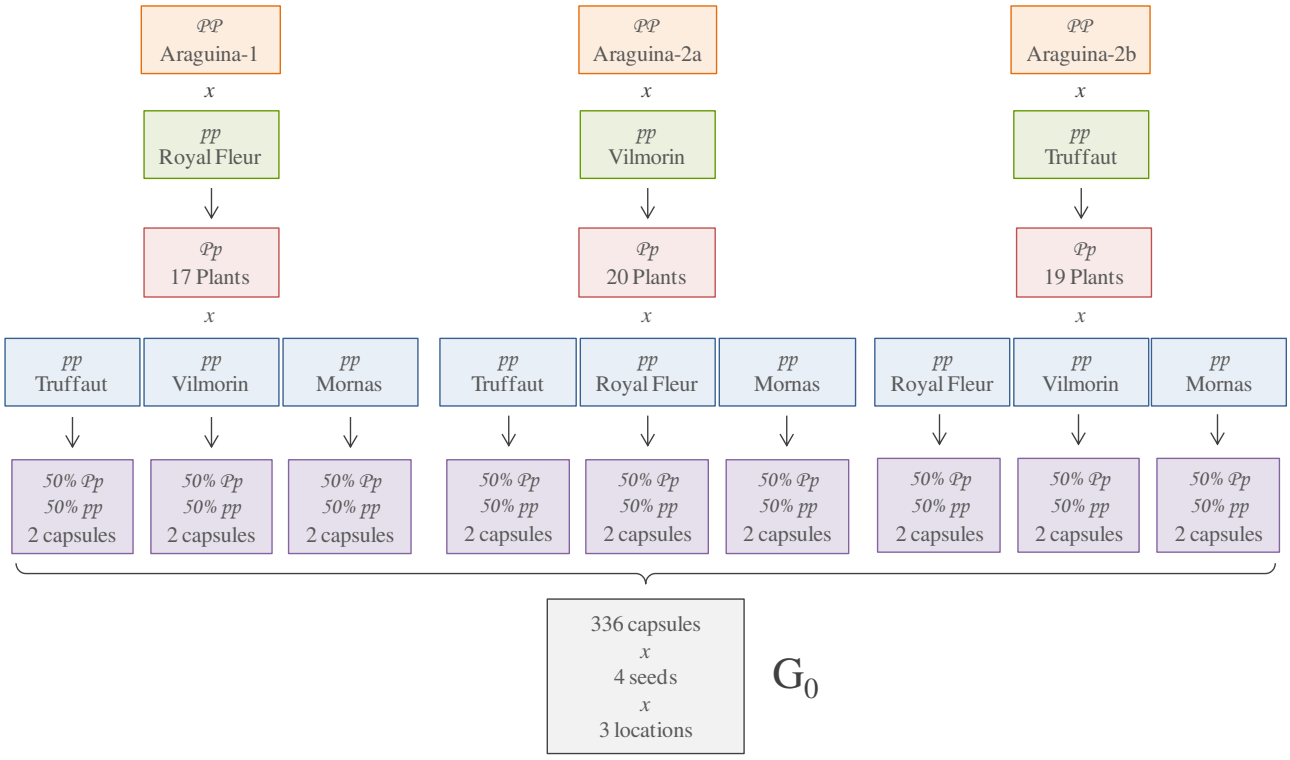

Figure 2. Diagram of the set-up for the G_0 seed production procedure for the experimental population.

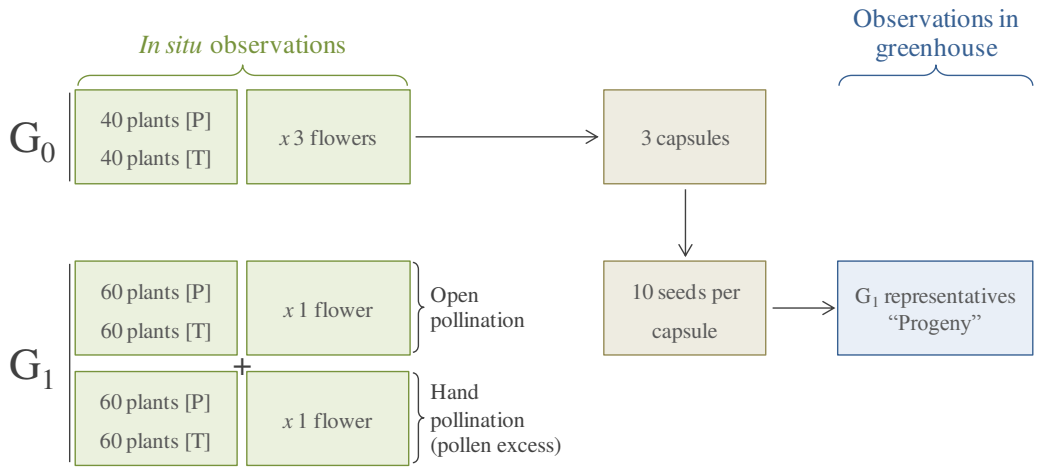

Figure 3. Diagram of the set-up for *in situ* and greenhouse observations on G_0, G_1 and progenies.

Morphological measurements and notations during flowering – G_0

In the spring 2012, during flowering time, forty G_0 plants of each morph at each of the three sites were randomly chosen and tagged for observation. To reduce micro-environmental heterogeneity effects, plants were chosen in couples (one [P] and one [T]) located side by side. By doing so we also avoided observing monomorphic patches. We were concerned that this could lead to a biased selection of [P] and [T] plants of similar stature, which in turn might affect the morphological measures on floral traits. Indeed no significant differences were observed between morphs for plant height in any of the replicates over the two generations (see *Differences between population replicates* section of Results). Therefore we did an independent test for plant height differences by randomly sampling 61 [P] and 61 [T] plants on the G_1 of MLN replicate. Again this test showed no significant differences between morphs ([P] 72.12 ± 11.64 cm, [T] 73.64 ± 11.52 cm, mean ± SD, $F_{(1, 120)} = 0.52$, $P = 0.47$).

Approximately a week after the population replicates began to flower a number of morphological traits were measured and/or recorded. At the plant level, on plants with at least three open flowers we recorded plant height, number of open flowers and number of formed capsules. For each plant three open flowers were chosen at random and a number of traits were measured and/or recorded at the flower level: perianth diameter, number of sepals and petals or total number of sepals and sepal-like organs, number of stamens and number of carpels. These characters were averaged per plant for later statistical analysis (see *Data analysis* section of Methods).

Morphological measurements and notations on capsules and seeds – G_0

In the summer 2012, upon capsule maturation, the total number of formed capsules in each of the 40 [P] and [T] plants was recorded and the three capsules corresponding to the three previously observed flowers on each plant were measured in length and diameter, and collected in individual bags. A capsule maturity trait was recorded based on the opening state (closed or open) which was later used to correct the seed number of open capsules. For each capsule the total seed weight and total number of seeds was recorded.

Insect observations – G_0

Insect behavior was studied by observing during 10 minute periods a couple of [P] and a [T] plant (when possible, the previously established [P] and [T] plant couples were used). For each 10 minute window we observed and registered the visit of each insect, the insect type (*Apis*, *Bombus*, or other) and the duration of the visit (**Figure 4**). A visit was considered as such from the moment an insect landed and stayed for more than a second on any flower of a plant under observation. Time of day and observed plant (if applicable) were also noted. At each site between 30 and 40 periods of 10 minutes were logged.

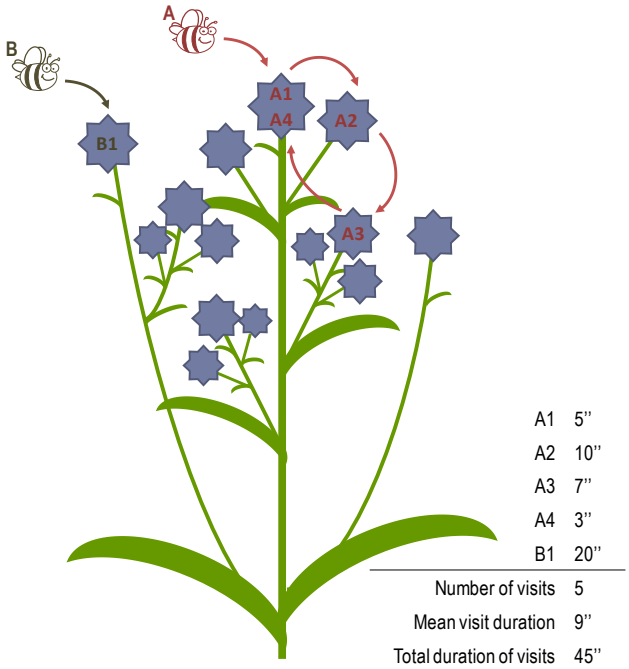

Figure 4. Schematic representation of insect behavior observation, notation method and variable interpretation.

Morphological measurements and notations on progeny

For each of the 40 [P] and [T] plants observed at each site, ten seeds were sampled randomly (4+3+3 seeds out of the three collected capsules per plant) and sown in greenhouse controlled conditions were a number of traits were measured, to assess a global vigor of each plant's progeny. The greenhouse design was organized in two blocks of 11 multi-pots. Each progeny lot of ten seeds was divided into subsets of five seeds, each being randomly sown in each block (**Figure 5**). Seeds were sown in a

mixture of 4:1 soil and vermiculite treated with Previcur fungicide, and watered every other day. A nutrient solution was added to watering once a week after germination onset. Greenhouse conditions were set for long day periods (16h day – 8h night) with a temperature of 23°C during the day and 18°C during the night and 60% relative humidity.

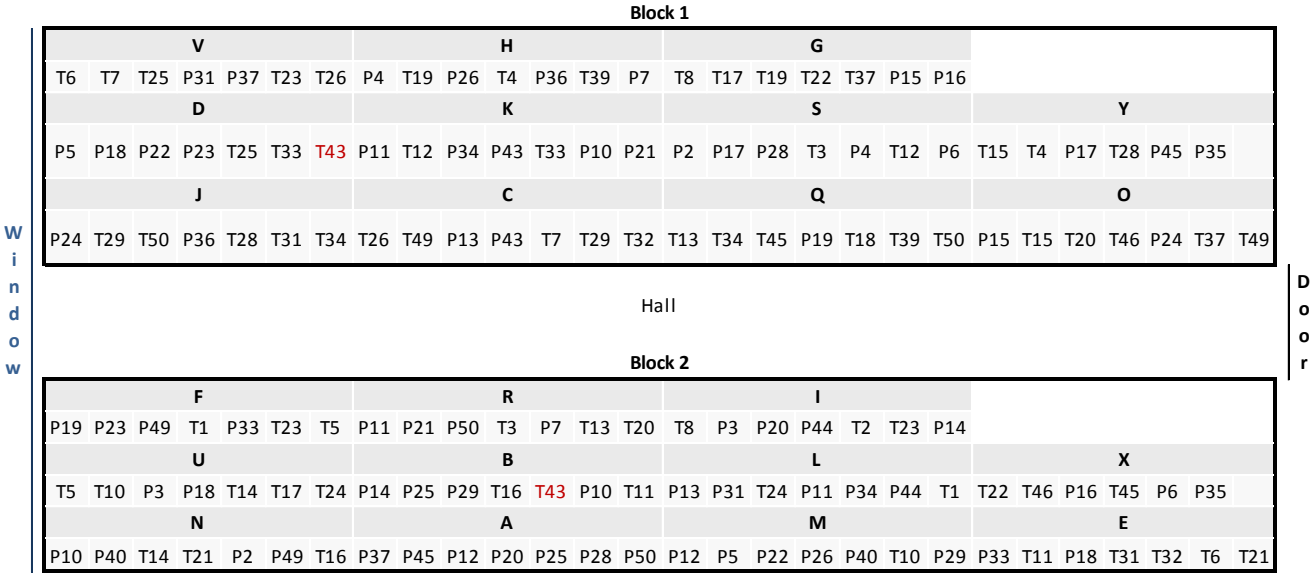

Figure 5. Example of experimental set-up for progeny study in greenhouse. A-Y: multi-pots. Each progeny, represented by the morph and number of mother plant (e.g. T43), was replicated twice, once in each block.

The first germinations marked the first day of observations (day 0). Every two days the experimental set up was inspected for seed germination. The dates and number of germinations for each G_0 mother plant were hence recorded. Three dates were chosen for observations on developing plants: 23 days after the onset of germination we counted the number of leaves and plant height (either the length of the longest leaf, before floral transition, or the length of the primary stem); 42 days after the onset of germination we measured plant height again; and an additional measure of plant height and leaf number was made for each plant 21 days after its germination date. When possible, towards the end of the observation periods, the presence of flowers was used to identify plant morph, [P] or [T].

Due to a difference in flowering time (i.e. some plants not flowering until a much later date) we also characterized floral morph through genotyping. At the end of the observation period, plant tissue was sampled from each germinated plant individually, for DNA extraction according to a protocol adapted

from the Dneasy 96 Plant Kit (QIAGEN, USA), and subsequent PCR genotyping for the presence/absence of the MITE insertion at the *NdAP3-3* locus (Chapter 1).

Morphological measurements and notations during flowering – G_1

In spring 2013, G_1 plants were observed during flowering time. Two sets of 60 [P] and 60 [T] plants were randomly chosen and tagged for observation at each of the three sites. The two sets of [P] and [T] plants were used in an open pollination *versus* hand pollination (pollen excess) experiment (**Figure 3**). A number of morphological traits were measured and/or recorded at the plant level: flower color, plant height, number of open flowers and number of formed capsules. For each plant a single flower at the pre-anthesis bud stage was selected and tagged for later observation. Flowering stage was observed and recorded from onset to end of anthesis over a time period of 8 to 10 days. During anthesis a number of morphological traits were measured and/or recorded at the flower level: perianth diameter, number of sepals and petals or total number of sepals and sepal-like organs. Flowers from the free pollination plant set were left untouched and allowed to reproduce freely whereas flowers from the pollen supplement set were pollinated with an excess of foreign pollen. Pollen addition was performed between the flowering stages *c* and *d* depicted in **Figure 6**, with pollen from neighboring plants of both morphs when available.

Morph-ratio determination

During the flowering period, both in the G_0 and G_1, we evaluated the proportion of each morph in the field plots on a number of plants randomly sampled along four different transects across the plots (between 50 and 100 plants per transect). This procedure was repeated by two independent experimenters and the results were pooled.

Data analysis

Morphological traits measured in the G_0 (2012) were averaged per plant (arithmetic mean of the three flowers or capsules). A number of capsules were collected at an open state which entailed some to

complete seed loss (empty capsules were collected on several occasions), therefore, seed number of open capsules was mathematically corrected. For each population replicate, seed number of closed capsules was regressed on its most correlated variable (capsule length for MAG and MLN and capsule diameter for CEF) and the obtained linear regression coefficients were then used to correct seed number on open capsules (*corrected seed number* = $a + b$ x *capsule size*). For the traits measured on the progeny (except germination rate, see below), because there was an effect of multi-pot position within the blocks, each mother's average value was obtained by adjusting for multi-pot effect on each trait using linear regression models. Three classes of multi-pots were determined based on the percentage of germinations per multi-pot (less than 45 %, between 45 % and 65 %, and more than 65 %) and for each trait the average for the multi-pot class was used as a co-variable in the model (*trait ~ mother plant + trait average of multi-pot class*). The partial linear regression coefficients of each model were used to estimate the adjusted means of each mother plant for the different traits. The germination rate or probability of germination of each mother plant progeny was calculated using a generalized linear model with a binomial distribution that included the average number of germinations per multi-pot class as a co-variable (*germination probability ~ mother plant + germination average of multi-pot class*). The canonical logit link function was used to transform the means into probabilities.

Differences between morphs in vegetative, floral, reproductive and progeny traits were assessed using multi-way ANOVA. Each time we controlled for population replicate and the interaction between morph and replicate. We analyzed data globally (at the population level) in the absence of a significant interaction between morph and replicate, or in case the interaction effect was significant but the same tendency was found among the population replicates (the same morph having higher or lower values across replicates). The effect of year was also accounted for in the analysis of floral traits for which measures for both G_0 and G_1 had been made. The effect of microenvironment (i.e. common location for the couples of [P] and [T] plants) was also added to the models for the analysis of vegetative, floral and reproductive traits.

Correlations between floral traits and between floral and insect behavior traits were analyzed using Pearson's product-moment correlation method on the global population data.

In order to identify floral traits under selection through correlation with fitness, we estimated individual relative fitness using two methods. The first is based on simple manipulations of trait values considered to be components of fitness, such as the number of capsules, the number of seeds and the seed mass. Hence we calculated the fitness variable *Seeds* as the number of capsules per plant multiplied by the mean number of seeds per capsule, and *Seed mass* as the number of capsules per plant multiplied by the mean seed mass per capsule. Because the original variables were previously standardized there was no need for transformation of the calculated variables (as confirmed by looking at their residues). The second method is based on the summarization of a range of reproductive traits using principal component analysis (PCA). From the following list of traits we extracted the two first principal components (*PC1* and *PC2*): plant height, number of capsules, capsule length, capsule diameter, seed number per capsule, seed mass per capsule, mean seed weight, germination rate of progeny and the different measures of progeny development. We first tested for differences of "absolute" fitness between morphs and population replicates using two-way ANOVA. We then estimated relative fitness variables by dividing individual fitness by the morph means in the population, as suggested by Lande and Arnold (1983). The relative fitness measures were then used to detect selection on floral characters using multiple regression models of relative fitness on five different floral traits (number of open flowers, flower diameter, number of perianth organs, number of stamens and number of carpels) according to Lande and Arnold's (1983) method. Prior to inclusion in the model, all floral traits were standardized. Linear regression models were used to detect linear (directional) selection, and non-linear selection (stabilizing or disruptive) was detected by performing non linear quadratic regression. Directional selection gradients (β) were estimated by performing multiple regression models including all floral traits as linear terms (*fitness ~ floral traits + morph + morph * floral traits*). Non linear selection gradients (γ) were obtained from complete models with both linear and quadratic terms (*fitness ~ floral traits + morph + (floral traits)*^{2} *+ morph * floral traits + morph * (floral traits)*^{2}). Interaction terms were included in each model to control for differences in

selection between morphs. Quadratic regression coefficients were doubled to obtain the corresponding nonlinear selection gradients (Stinchcombe *et al.*, 2008).

Morph-ratio was studied using Pearson's chi-squared test of goodness of fit to determine whether [P] and [T] proportions differed from 50-50 in G_0, and from the proportions expected hypothetical reproduction scenarios in G_1. The chi-squared test of independence was used to determine whether the [P] and [T] proportions differed among the population replicates. If the null hypothesis could not be rejected the proportions of the global population were used in the goodness of fit analysis.

All data analyses were conducted using the statistical program R 3.0.1 (R Development Core Team, 2013). The package FactoMineR was used for principal component analysis and the package ggplot2 was used for plot construction.

Results

Floral traits and morphology associated with reproduction mode

Our study of floral morphology and anthesis confirmed the overall pattern of style and stamen movements previously described in the literature. However, while previous authors have emphasized the role of those movements in potentiating the contact between pollinators and the stigmas (Toxopéus, 1927; Zhao *et al.*, 2011), in our observations it is also clearly visible that the spiral downward movement of the styles onto the stamens can lead to self-pollination. At the earliest stages of floral anthesis (**Figure 6***a* and *b*), styles are erect and stamens remain upright and tightly packed around the gynoecium. In subsequent stages (**Figure 6***c*) stamens become outspread, from outermost to innermost, so that at later stages (**Figure 6***d* and *e*) anthers are spread apart. Meanwhile the styles acquire a curved appearance and twist longitudinally exposing the style crests on several planes. This downwards movement continues until styles and anthers come into contact (**Figure 6***d* and *e*). Once a pollen load is deposited on the stigmatic surface, whether by a pollinator or by contact with a plant own anther, the style will start to unfold, straightening back up and slightly untwisting before returning to a position homologous to the initial situation (**Figure 6***a* and *b*). At those later stages the number of anthers is reduced as a consequence of their abscission, which along with perianth wilting and fruit inflation becomes a defining feature in distinguishing a recently open flower from a pollinated one.

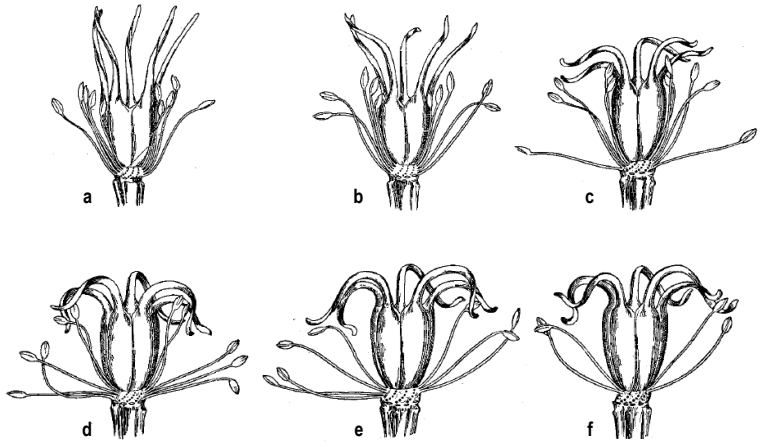

Figure 6. Sequence of style and stamen movements during anthesis of *Nigella damascena* flowers. Figure adapted from Toxopéus, 1927.

Pollinator identification and behavior

We studied insect behavior in relation to the two floral morphs by direct *in situ* observations at the field plots (summer 2012). *Nigella damascena* flowers are frequently visited by an array of generalist insects, most commonly hymenopterous. Among these, only the bees (genus *Apis*) and bumble bees (genus *Bombus*) exhibited a constant and purposeful pattern of visitation during our observations. This pattern was closely associated with the presence of the nectariferous petals ([P] morph), as insects were seen to land on the sepals and proceed to go around the flower collecting nectar from each petal (**Figure 7**). Although the nectar collecting behavior seems to concur with the theories of style and stamen movements, we found that insects visited flowers throughout the anthesis stages and not only at a perceived "optimal" stage for pollination (i.e. panels *a* and *b vs.* panels *c* to *f*, **Figure 6**). Perhaps the most striking observation of insect behavior was that the nectar collecting visitation pattern (landing and tour around the flower) was never seen in the [T] morph apetalous flowers. What is more, pollinating insects were rarely seen to land on [T] flowers. Both bees and bumble bees seemed able to perceive the absence of nectar without approaching the flowers and divert the course mid air to another plant. During the summer 2013 we informally observed an additional visitation behavior by members of the *Apis* and *Bombus* genera, which was focused on the collection of pollen, and occurred both in [P] and [T] morph flowers.

Figure 7. Photographic series depicting the bee visitation pattern of [P] morph flowers (possessing petals).

In the summer 2012, insect behavior was also quantitatively studied (**Figure 4**). On average, for a 10 minute time period, [P] plant flowers were visited significantly more often and for longer periods than [T] plants (**Table 1**). The mean duration of visits to a flower in [T] plants was close to zero while in [P] plants it was about 12 seconds. This difference closely mirrors the nectar collecting focused behavior observed in 2012.

Table 1. Insect (*Apis* and *Bombus*) visitation traits, means ± SD by morph. Mean values are pooled among the three population replicates. Means in bold correspond to the higher value when significant. $F_{(\text{degrees of freedom})}$: statistics for morph comparison, from a model considering the effects of morph, population replicate and their interaction. All three models for different traits had the same degrees of freedom (shown in Morph column). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Morph	Number of visits during 10' period	Mean duration of each visit	Total duration of visits in the 10' period
[P]	**7.82** ± 6.56	**11.59** ± 6.77	**88.79** ± 77.72
[T]	2.60 ± 2.02	0.53 ± 1.59	1.03 ± 2.62
$F_{(1, 151)}$	40.87 ***	161.49 ***	80.76 ***

We also tested for the influence of different floral traits on insect visitation behavior of [P] plants using Pearson's product moment correlation analysis (**Table 2**). The number of perianth organs has a significant positive effect both on the number of visits and the total duration of visits. Additionally flower diameter is positively correlated with the total duration of visits to a plant in a 10 minute period.

Table 2. Pearson's product moment correlation coefficients between floral traits and insect behavior traits measured on [P] plants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Number of visits during 10' period	Total duration of visits in the 10' period	Mean duration of each visit
Open flowers	0.133	0.121	-0.063
Flower diameter	0.125	0.223 *	0.164
Perianth organs	0.274 *	0.270 *	0.025
Stamens	0.111	0.205	0.255 *

Differences between population replicates

Population replicates differed visibly in plant stature and architecture (**Figure 8**). Both in the 2012 and 2013 observations, the MLN replicate plants were significantly taller than the plants from the southern

replicates (mean ± SD: MLN 67.63 ± 12.32 cm, CEF+MAG 40.39 ± 9.87 cm, $F_{(1,\ 923)} = 1321.3$, $P < 0.001$). This is likely because MLN has a richer soil both in nutrients and water, while the CEF and MAG soils are typically dry and destitute. Additionally plant height at MLN increased significantly in the second year (2012: 51.65 ± 7.67 cm, 2013: 72.31 ± 9.07 cm, $F_{(1,\ 307)} = 300.23$, $P < 0.001$). This difference between years is likely due to a stress caused by the transplanting in the first year. Plants at CEF and MAG also differ in appearance between the two replicates, MAG plants being significantly shorter than CEF (CEF 45.62 ± 9.42 cm, MAG 35.02 ± 7.04 cm, $F_{(1,\ 478)} = 265.79$, $P < 0.001$). In addition, in 2012 MAG plants had thick stems and produced secondary ramifications, whereas plants from CEF were thinner and often produced the primary stem alone. The spindly stature of CEF plants observed in 2012 could have been a consequence of the presence of competing species in high density in that terrain, such as the populous poppy (*Papaver rhoeas*), that was absent from the MAG population.

Figure 8. Schematic representation of plant architecture in the three population replicates. a. CEF, b. MAG, c. MLN.

Despite significant differences between population replicates plant height does not differ significantly between morphs within replicates or in the ensemble over the two years ([P] 49.48 ± 16.69 cm, [T] 49.50 ± 16.84 cm, $F_{(1,\ 923)} = 0.0004$, $P = 0.99$). Differences in floral characters among the

population replicates are likely a reflection of the differences of plant stature and architecture which are here interpreted as measures, or under the influence, of plant vigor.

Differences between [P] and [T] – Floral traits

During the flowering period different floral traits were observed, including flower size and the number of each type of organs (**Figure 9**). Along with the morphological differences between morphs mentioned before (absence of petals and production of a series of sepal-like organs in [T] morph) we observed a significant difference in the total number of perianth organs, with the total number of sepals plus sepal-like organs in [T] morph being greater than that of sepals plus petals in [P] flowers (**Table 3**). This significant difference was true for all population replicates in both years. Stamen number also differs between morphs with [P] flowers showing a superior number than [T], while carpel number did not show any differences between morphs (**Table 3**). Despite the fact that [P] showed a greater number of stamens than [T], the total number of floral organs (perianth + stamens + carpels) in the [T] morph flowers is significantly greater than in the [P] morph (**Table 3**). Flower diameter measured at the level of the perianth is significantly different between morphs when all replicates are analyzed together (**Table 3**), and for the MAG replicate in 2013. Additionally the same tendency can be seen every time, with [T] flowers being larger than [P].

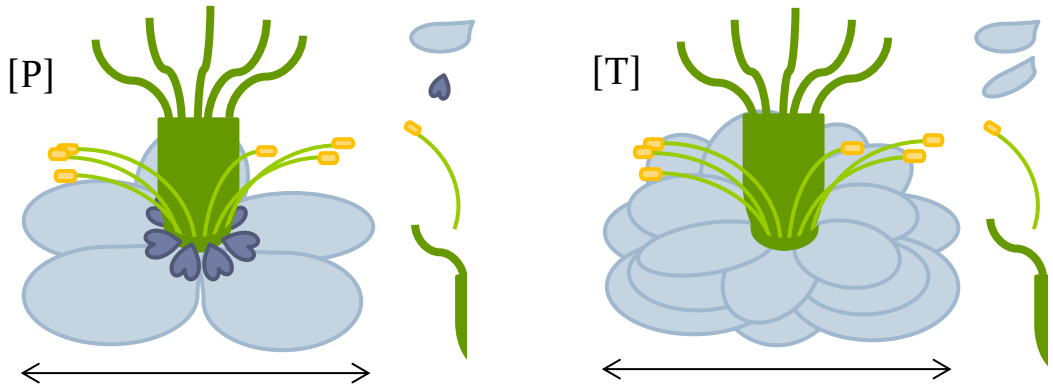

Figure 9. Schematic representation of [P] and [T] morph flowers and the floral traits measured/counted for each morph. Left: [P] morph with sepals (light blue), petals (dark blue), stamens (light green and yellow) and carpels (dark green). Right: [T] morph with sepals and sepal-like organs (light blue), stamens and carpels. Flower diameter was measured as indicated by the double arrows.

Table 3. Floral traits, means ± SD by morph. Values pooled among the three population replicates and, for perianth organs, between the two years. Means in bold correspond to the higher value when significant. $F_{(\text{degrees of freedom})}$: statistics for morph comparison from a model considering the effects of morph, population replicate, year (for perianth and diameter), microenvironment and the interaction between morph and population replicate. Residual degrees of freedom (r.d.f) are indicated for each trait. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Morph	Perianth	Stamens	Carpels	Total floral organs	Diameter (mm)
[P]	14.01 ± 2.08	**46.23** ± 6.84	5.08 ± 0.39	66.08 ± 7.47	38.49 ± 7.42
[T]	**21.83** ± 6.92	41.46 ± 6.47	5.04 ± 0.66	**73.69** ± 12.61	**39.76** ± 7.10
$F_{(1, \text{r.d.f.})}$	872.4 $_{(635)}$ ***	31.3 $_{(211)}$ ***	0.27 $_{(211)}$	47.4 $_{(211)}$ ***	30.8 $_{(632)}$ ***

Correlations between floral traits were determined using Pearson's product-moment correlation analysis (**Table 4**). All traits are more or less positively correlated to each other in both morphs, bigger flowers supporting greater organ numbers. The absence or poor correlation of the number of carpels with flower diameter and stamen number is not of particular relevance as carpel number is a discontinuous trait with small variance.

Table 4. Pearson's product-moment correlation coefficients among floral traits for [P] (below the diagonal) and [T] (above the diagonal) morphs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Flower diameter	Perianth organs	Stamens	Carpels	Organs (total)
Flower diameter		0.197 *	0.439 ***	-0.034	0.387 ***
Perianth organs	0.319 ***		0.019	0.361 ***	0.858 ***
Stamens	0.517 ***	0.216 *		-0.039	0.527 ***
Carpels	0.233 *	0.316 ***	0.189 *		0.332 ***
Organs (total)	0.555 ***	0.432 ***	0.972 ***	0.294 **	

Differences between [P] and [T] – Capsule and seed production

Upon capsule maturation the total number of capsules produced on the previously observed G_0 plants was counted and a series of measurements were made on the three capsules corresponding to previous flower observations (**Figure 10**). Those capsules were collected and the respective seed sets were studied. The number of capsules produced per plant is not significantly different between morphs whether tested in the global population or within replicates. [T] morph plants produce bigger capsules than [P], whether this is measured in length or in diameter (**Table 5**). The greater capsule size of [T] plants seems to reflect on seed production, as [T] capsules have on average significantly more seeds (**Table 5**). This difference, however, does not echo on total seed mass, which does not differ

significantly between morphs (**Table 5**). The absence of a significant difference of total seed mass per capsule is possibly a consequence of the significant difference of mean seed weight in favor of [P] morph plants (total seed mass per capsule divided by the number of seeds per capsule, **Table 5**).

Table 5. Capsule and seed production traits means ± SD per morph. Values pooled among the three population replicates. Means in bold correspond to the higher value when significant. $F_{(\text{degrees of freedom})}$: statistics for morph comparison, from a model considering the effects of morph, population replicate, microenvironment and the interaction between morph and population replicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Morph	Capsule length (mm)	Capsule diameter (mm)	Seeds per capsule	Total seed mass per capsule (mg)	Mean seed weight (mg)
[P]	20.35 ± 3.17	16.88 ± 2.06	56.32 ± 17.61	135.93 ± 74.75	**2.55** ± 0.82
[T]	**21.62** ± 3.31	**17.66** ± 2.21	**68.75** ± 20.82	145.53 ± 84.65	2.15 ± 0.88
$F_{(1, 211)}$	8.93 **	7.28 **	25.20 ***	0.54	17.83 ***

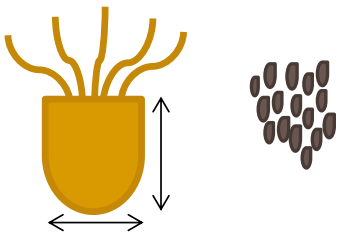

Figure 10. Schematic representation of a capsule and seed set. Capsule length and diameter measures indicated by double vertical and horizontal arrows, respectively.

Differences between [P] and [T] – Progeny development and vigor

For each plant observed in 2012 (generation G_0) a progeny of 10 seeds was sown in greenhouse, under controlled conditions. For each progeny the germination outcome and plant vigor were evaluated. There is no significant difference between the germination success of seeds descending from [P] or [T] morph plants, whether this is measured as a germination rate ([P] 0.79 ± 0.15, [T] 0.78 ± 0.16, $F_{(1;216)} = 0.37$, $P = 0.55$) or timing (days after global germination onset: [P] 7.81 ± 4.86, [T] 7.29 ± 4.89, $F_{(1;216)} = 0.62$, $P = 0.25$). Among the vigor traits studied in the progeny only the number of leaves 21 days after germination (d.a.g.) and 23 days after the onset of germination (d.a.o.g.) was significantly different between the progenies of [P] and [T] morph plants (**Table 6**).

Table 6. Progeny traits, means ± SD by morph of parent plant (mother). Means in bold correspond to the higher value when significant. $F_{(\text{degrees of freedom})}$: statistics for morph comparison, from a model considering the effects of morph, population replicate and the interaction between the two. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Parent Morph	Number of leaves at 21 d.a.g.	Plant height at 21 d.a.g. (cm)	Number leaves at 23 d.a.o.g.	Plant height at 23 d.a.o.g. (cm)	Plant height at 42 d.a.o.g (cm)	Difference of height between the 42nd and 23rd d.a.o.g. (cm)
[P]	**5.93** ± 0.82	114.44 ± 12.25	**5.46** ± 1.28	105.69 ± 15.74	195.00 ± 38.38	89.30 ± 34.70
[T]	5.54 ± 0.76	113.11 ± 13.23	5.08 ± 1.25	104.14 ± 18.60	196.51 ± 44.01	92.37 ± 39.72
$F_{(1, 216)}$	13.07 ***	0.60	4.73 *	0.45	0.07	0.37

Within the entire progeny set four types of progeny can be distinguished: the [P] descendants of [P] morph mothers (P-P, $n = 352$), the [T] descendants of [P] morph plants (P-T, $n = 365$), the [T] descendants of [T] morph plants (T-T, $n = 652$), and the [P] descendants of [T] morph plants (T-P, $n = 13$). By comparing the two progeny morphs descending from [P] mother plants (P-P *vs.* P-T) we should be able to assess the effect of the locus *P* on the overall plant vigor whereas the equivalent comparison for [T] mother plants (T-P *vs.* T-T) should reveal the combined effects of *P* locus and inbreeding. Indeed, because G_0 [T] plants are homozygous, a [P] offspring from [T] mother plants necessarily results from outcrossing. However as we saw before, these plants are very little visited by insects therefore the majority of the [T] offspring from [T] mother plants is very likely the result of selfing. The comparison of the [P] descendants from [P] *versus* [T] mother plants (P-P *vs.* T-P) and the comparison of [T] descendants from [P] plants *versus* [T] mother plants (P-T *vs.* T-T) could provide an indication of the effect of the mother's morph on progeny vigor although a potential effect of inbreeding may also be present in the progeny of [T] plants (T-T). The number of leaves at 21 days after germination was significantly greater in [P] morph plants when comparing [P] *vs.* [T] descendants independent of the mother's morph. Additionally, both the number of leaves at 21 days after germination and the number of leaves 23 days after global germination onset were significantly greater for the descendants of [P] plants, in the comparison P-T *vs.* T-T (

Supplementary table 1).

Estimating fitness

We estimated four different components of fitness: number of seeds per plant (capsules per plant *x* seeds per capsule - *Seeds*), seed mass per plant (capsules per plant *x* seed mass per capsule – *Seed mass*), and the two first principal components of a PCA performed on the vigor, reproductive and progeny traits measured on both morphs. The first principal component (*PC1*) explains 36.83% of the total variance and is mainly weighed in by capsule size, seed mass per capsule and mean weight of seeds, while traits associated to plant and progeny vigor (i.e. plant height and capsule number, seeds per capsule, progeny height and leaf number) contribute to the second principal component (*PC2*) which explains 19.31% of the total variance (**Supplementary table 2**). Fitness measures hence obtained were tested for differences between morphs and population replicates. All fitness components showed significant differences among population replicates but no interaction between morph and replicate were detected, so the following analyses were done on the global population. The second principal component was the only measure to show a significant difference between the two morphs (**Table 7**). After this initial analysis, each of the four fitness components measures were transformed into relative fitness measures by dividing the individual values by the morph means in the population.

Table 7. Mean values for four fitness estimates in the two floral morphs [P] and [T], and significance level of the morph and population replicate (Rep) effects, and the interaction of both. Means in bold correspond to the higher value when significant. * *P* < 0.05, ** *P* < 0.01, * *P* < 0.001.**

x	***Seeds***	***Seed mass***	***PC1***	***PC2***
[P]	0.275	0.357	0.043	-0.38
[T]	0.403	0.481	-0.048	**0.43**
Rep	*	**	***	***
Morph				***
Rep*Morph				

Detection of selection on floral traits

Selection on floral traits was examined using multiple regression analysis of the different measurements of relative fitness on five floral traits (**Table 8**). We found significant evidence for directional selection on different traits according to the measurement of relative fitness used. The

significant effects found in the global population analysis were also found in the analysis by population replicates, or at the least the same tendency was found.

Table 8. Linear (β) and quadratic (γ) selection gradients for the different floral traits as determined by the different relative fitness measures. Values in bold correspond to significant gradients. * *P* < 0.05, ** *P* < 0.01, * *P* < 0.001.**

All replicates	***Seeds***		***Seed mass***		***PC1***		***PC2***	
Linear	β	Interaction with morph	β	Interaction with morph	β	Interaction with morph	β	Interaction with morph
	$R^2 = 0.08$		$R^2 = 0.08$		$R^2 = 0.15$		$R^2 = 0.40$	
Flower diameter	-0.47	0.56	0.34	-0.01	**14.73** **	**-31.90** ***	**-2.17** ***	**2.76** ***
Perianth organs	**5.64** **	**-5.05** *	**4.60** **	**-4.45** **	-43.22	49.58	-2.88	**4.60** *
Stamens	-0.01	0.62	-0.14	-0.06	-0.16	-7.12	-0.28	0.87
Carpels	-0.48	0.26	-0.31	0.45	1.96	-2.15	-0.31	0.39
Open flowers	-0.62	0.53	**-0.67** *	**0.77** *	4.83	-7.30	0.23	0.28
Morph	**-3.62** *		**-3.14** **		20.02		0.84	

Quadratic	γ	Interaction with morph	γ	Interaction with morph	γ	Interaction with morph	γ	Interaction with morph
	$R^2 = 0.21$		$R^2 = 0.16$		$R^2 = 0.18$		$R^2 = 0.45$	
Flower diameter	0.84	-0.60	**1.18** **	-1.02	-4.58	18.56	-0.04	0.10
Perianth organs	-6.10	6.60	-11.46	11.84	104.86	-101.36	14.70	-15.88
Stamens	0.44	1.20	0.06	0.32	-1.30	1.10	-0.06	0.50
Carpels	0.46	-0.10	0.44	-0.26	4.58	-5.06	0.12	0.12
Open flowers	0.28	-0.82	0.06	-0.40	-4.74	0.62	-0.42	0.40

A positive linear correlation between relative fitness and flower diameter was detected using both *PC1* and *PC2* as relative fitness measures (**Table 8**). Because there was a significant effect of morph interaction with flower diameter on both fitness traits we also performed both multiple regressions by morph. When analyzed separately for each morph, *PC1* reveals a significant positive correlation with flower size in [P] morph ($\beta = 14.73$, $P < 0.05$) and a significant negative correlation in [T] morph ($\beta = -17.16$, $P < 0.01$) (**Figure 11**). When regressing *PC1* in [T] morph floral trait values we also found a significant positive non linear correlation with flower diameter ($\gamma = 13.56$, $P < 0.05$). In accordance with the opposing signs of the morph interaction coefficients in both fitness variables, *PC2*

reveals the inverse tendency of *PC1*. Analysis of *PC2* by morph shows a significant negative correlation with flower diameter for the [P] morph ($\beta = -2.17$, $P < 0.001$), while no significant correlation was found in [T] morph plants (**Figure 11**). Finally, flower diameter was also shown to be significantly correlated with *Seed mass* in a nonlinear relation (**Table 8**). Despite the absence of a significant interaction with morph, upon analysis by morph and graphical representation, we found that the nonlinear correlation is significant only for [P] morph plants ($\gamma = 0.59$, $P < 0.05$) (**Figure 11**).

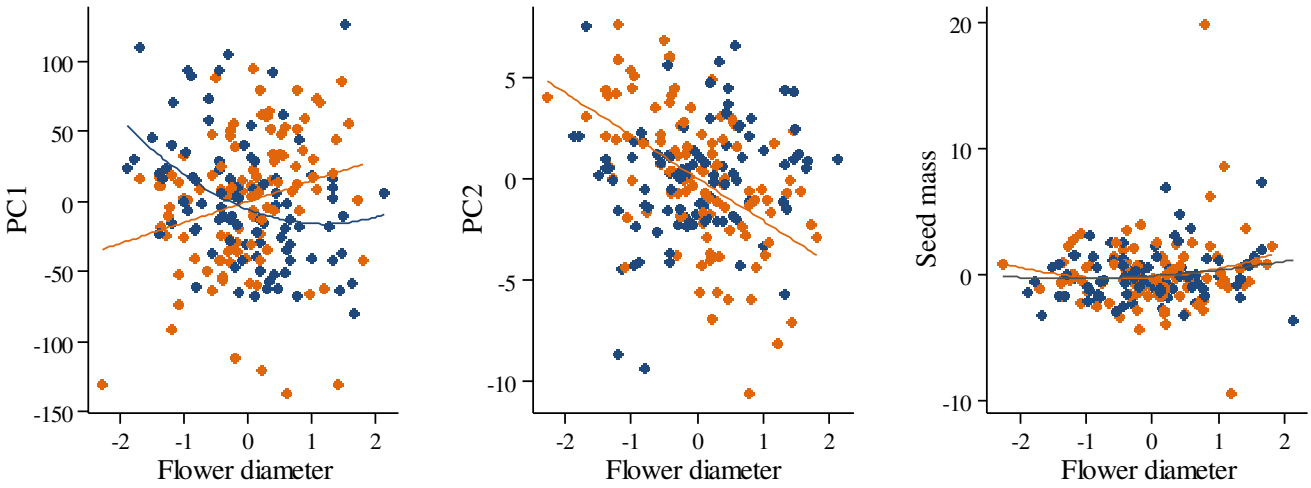

Figure 11. Correlation between flower diameter and relative fitness as estimated by *PC1*, *PC2* and *Seed mass*, in [P] (orange) and [T] (blue) morph plants. y-axis shows the fitness trait residualized for all floral traits except flower diameter in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph), and x-axis shows flower diameter residualized for the remaining floral traits in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph). *PC1* shows the linear regression line for the [P] morph and the quadratic regression curve for the [T] morph. *PC2* shows the linear regression line for the [P] morph. *Seed mass* shows the quadratic regression curve for both morphs together (black) as well as for the [P] morph alone.

Both the *Seeds* and *Seed mass* relative fitness variables revealed positive linear correlations with the number of perianth organs (**Table 8**), but there was also a significant morph interaction with floral trait, therefore analysis was performed by morph. Indeed, a significant positive correlation of *Seed* and *Seed mass* with the number of perianth organs was found only for [P] morph plants (*Seed*: $\beta = 5.64$, $P < 0.05$, *Seed mass*: $\beta = 4.60$, $P < 0.05$) (**Figure 12**).

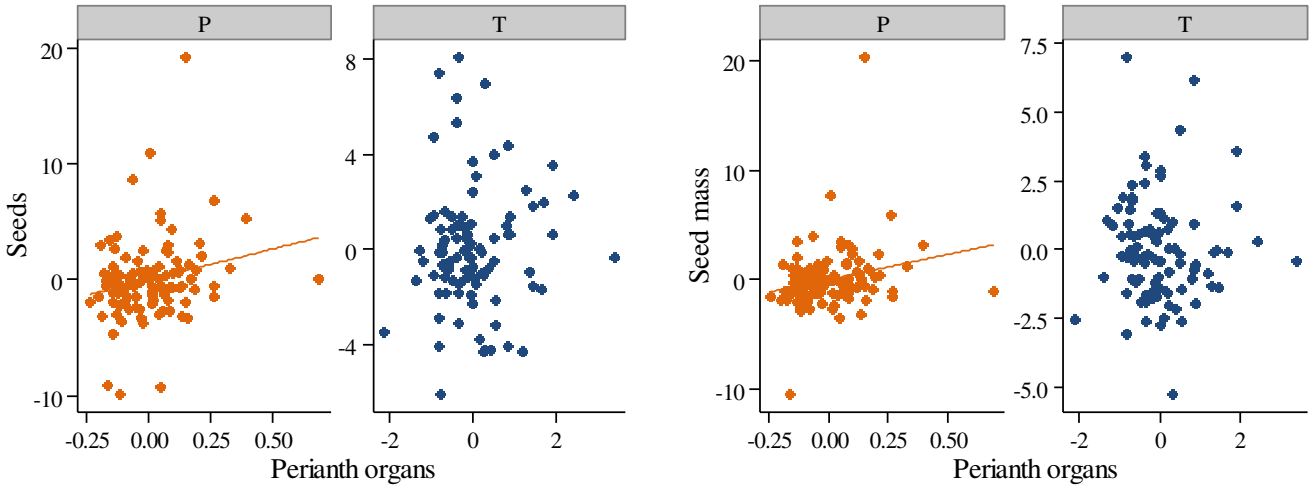

Figure 12. Correlation between perianth organ number and *Seeds* or *Seed mass*, in [P] (orange) and [T] (blue) morph plants. y-axis shows the fitness trait residualized for all floral traits except perianth organ number in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph), and x-axis shows perianth organ number residualized for the remaining floral traits in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph). Linear regression lines are only shown for the [P] morph.

PC2 revealed a significant interaction of morph with perianth organ number in the linear model, despite no significant main effects. Indeed when analyzed by morph, *PC2* was significantly correlated with perianth organ number for [T] morph plants only, with significant linear ($\beta = 1.72$, $P < 0.001$) and nonlinear ($\gamma = -0.59$, $P < 0.05$) coefficients. While a positive correlation is visible in the graphical representation of this regression, evidence for nonlinear selection is again very weak (**Figure** 13).

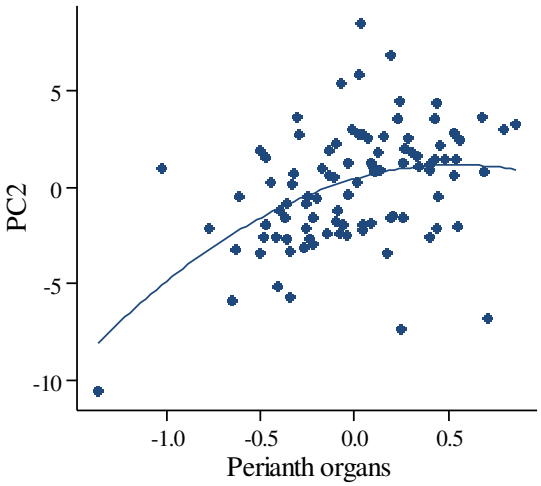

Figure 13. Regression of the fitness estimate *PC2* on perianth organs number of [T] morph plants. y-axis shows the fitness trait residualized for all floral traits except perianth organ number in a complete multiple regression model (including linear terms, quadratic terms), and x-axis shows perianth organ number residualized for the remaining floral traits in a complete multiple regression model (including linear terms, quadratic terms).

We also observed a significant, although moderate, effect of the number of open flowers on *Seed mass*, and an interaction with morph (**Table 8**). The significance of the linear correlation of *Seed mass* with the number of open flowers is a reflection of the significant negative correlation for [P] morph ($\beta = -0.67$, $P < 0.05$) (**Figure 14**).

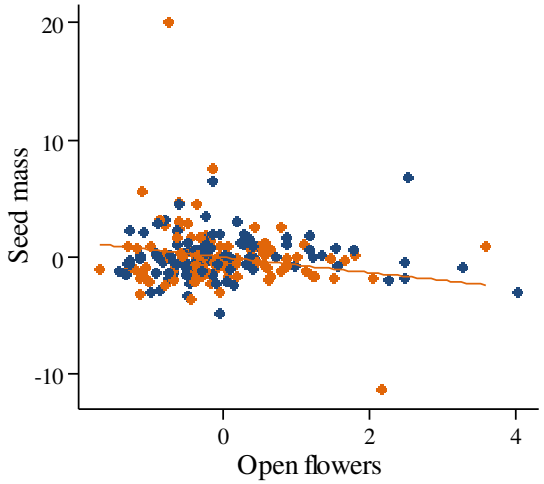

Figure 14. Correlation between the number of open flowers and *Seed mass*, in [P] (orange) and [T] (blue) morph plants. y-axis shows the fitness trait residualized for all floral traits except flower number in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph), and x-axis shows flower number residualized for the remaining floral traits in a complete multiple regression model (including linear terms, quadratic terms, and their interaction with morph). Only the [P] morph values were used to plot the linear regression line.

Morph-ratio

We determined the morph-ratio in the field plots (G_0 and G_1) and in the greenhouse set-up (progenies of G_0). The outcome of the G_0 seed lots sown in the field plots showed a significant bias to the expected 50-50 proportion of [P] and [T] plants, in all the population replicates (**Table 9**). The proportions in the different replicates do not differ significantly from each other ($\chi^2_{(2)} = 0.86$, $P = 0.65$), so the proportions for the global population were kept for further analysis.

Table 9. G_0 morph-ratios in the three population replicates. $\chi^2_{(\text{degrees of freedom})}$: statistics for the goodness of fit chi-squared test for the hypothesis that [P] and [T] have equal proportions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

G_0	CEF	MAG	MLN	Global population
[P]	88	83	81	252 (34.5%)
[T]	161	174	144	479 (65.5%)
$\chi^2_{(1)}$	21,40 ***	32,22 ***	17,64 ***	70.49 ***

Based on our observations of insect behavior and visitation patterns in the G_0 flowers, we hypothesized that [P] morph G_0 plants reproduced mainly via intra-morph crosses. From this hypothesis, seeing as all G_0 [P] plants are heterozygous *Pp*, we expected the progeny of [P] plants to be composed of 75% [P] and 25% [T] morph plants. However, because both selfing and intra-morph

crosses produce those same theoretical proportions of [P] and [T] descendants from [P] morph plants, we cannot test for an exclusively intra-morph reproduction mode specifically but only for a general conformity to those proportions. In all replicates, as well as in the global population, the frequencies in [P] morph progenies differed significantly from those expected under an intra-morph reproduction regime (**Table 10**).

The limited number of pollinator visitations on [T] morph flowers led us to hypothesize that reproductive success of G_0 [T] plants depended almost exclusively on self-pollination. Therefore, we expected their progeny to be exclusively [T]. Indeed, we found very few [P] descendants in [T] morph progenies, suggesting that [T] morph plants only do a limited amount of out-crossing (**Table 10**).

Because pollinating insects visited mostly [P] flowers, we assumed that the majority of pollen transported by pollinators derived from the [P] gene pool (composed of 50% *P* and 50% *p*). By doubling the proportion of [P] morph descendants in the [T] morph mothers' progenies, we thus estimated that [T] morph plants perform from at least a minimum 2 to 6% of out-crossing (**Table 10**).

Table 10. Morph-ratios in the progenies of G_0 [P] and [T] plants, by population replicates and for the global population. $\chi^2_{(\text{degrees of freedom})}$: statistics for the goodness of fit chi-squared test for the hypothesis of intra-morph reproduction for [P] morph plants (expected frequencies 0.75 [P] and 0.25 [T]). A minimal cross-pollination rate for [T] G_0 plants was calculated using twice the proportion of [P] morph descendants in [T] morph progenies. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mother	Progeny	CEF	MAG	MLN	Global population
[P]	[P]	117	111	137	365 (48.0%)
	[T]	107	126	162	395 (52.0%)
H_0: 1/4 [P], 3/4 [T]	$\chi^2_{(1)}$	88.60 ***	60.27 ***	69.12 ***	271.56 ***
[T]	[P]	6	3	5	14 (2.0%)
	[T]	187	224	260	671 (98.0%)
Minimal cross-pollination rate:		6.2%	2.6%	3.8%	4.2%

Based on our previous hypotheses for [P] and [T] plants reproduction modes, morph frequencies in the G_1 might be expected to follow certain proportions. Namely, if [P] plants reproduce among each other and [T] plants reproduce exclusively via self-pollination, and in the absence of differential selection between morphs, we could expect approximately a 1:4 ratio of [P] and [T] plants in the G_1 (**Table 11**).

Alternatively, we might consider using the proportions of [P] and [T] morph observed in the progenies of G_0 plants to predict morph frequencies in the G_1 (**Table 12**).

Table 11. Expected proportions of [P] and [T] plants in the G_1 assuming an intra-morph reproduction for [P] plants and self-pollination for [T] plants and absence of selection.

G_0 Morph – Genotype – Frequency:	[P] – Pp – 34.5%		[T] – pp – 65.5%	
Descendance Morph – Genotype:	[P] – PP, Pp	[T] – pp	[P] – Pp	[T] – pp
Theoretical proportions:	3/4	1/4	0	1
G_1 Morph frequencies:	f[P] = 0.345 * 3/4 + 0.655 * 0 = 0.259			
	f[T] = 0.345 * 1/4 + 0.655 * 1 = 0.741			

Table 12. Expected proportions of [P] and [T] plants in the G_1 assuming the morph-ratios observed in the progenies of G_0 [P] and [T] plants.

G_0 Morph – Genotype – Frequency:	[P] – Pp – 34.5%		[T] – pp – 65.5%	
Progenies Morph – Genotype:	[P] – PP, Pp	[T] – pp	[P] – Pp	[T] – pp
Observed progeny proportions:	48.0%	52.0%	2%	98%
G_1 Morph frequencies:	f[P] = 0.345 * 0.48 + 0.655 * 0.02 = 0.18			
	f[T] = 0.345 * 0.52 + 0.655 * 0.98 = 0.82			

The morph ratios observed in 2013 in the three G_1 population replicates, as well as globally, were compared to both hypotheses using chi-squared tests for goodness of fit (**Table 13**). In all population replicates as well as in the global population we could not reject the initial hypothesis for reproduction modes based on insect behavior, as [P] and [T] frequencies did not differ significantly from the 25.9 % – 74.1 % expected proportions. The second hypothesis, based on the observed morph-ratio in the greenhouse studied progenies of G_0 plants, was clearly rejected in two of the replicates as well as in the global population.

Table 13. G_1 morph-ratios in the three population replicates and in the global population. $\chi^2_{(\text{degrees of freedom})}$: statistics for the goodness of fit chi-squared test for the two hypotheses on the expected G_1 proportions of [P] and [T] morph plants. * *P* < 0.05, ** *P* < 0.01, * *P* < 0.001.**

G_1	CEF	MAG	MLN	Global population
[P]	176	193	63	432 (25.4%)
[T]	434	617	218	1269 (74.6%)
Intra-morph reproduction hypothesis (Table 11)				
$\chi^2_{(1)}$	2.77	1.81	1.77	0.22
Observed progeny morph-ratio hypothesis (Table 12)				
$\chi^2_{(1)}$	48.67 ***	18.63 ***	3.72	63.05 ***

Discussion

Despite their rare occurrence, and the likely low fitness that accompanies such transformations, natural polymorphisms of homeotic nature may be an invaluable tool in understanding the evolution of floral diversity. As pointed out before, here and elsewhere (Hintz *et al.*, 2006; Theißen, 2010), homeotic transitions are believed to have occurred several times during floral evolution, and often homeotic mutants observed today resemble common character state transitions between major flowering lineages. Therefore, studying those rare instances where natural homeotic mutants are successful in natural conditions and understanding the ecological mechanisms behind such success is of crucial importance to understanding the potential role of homeotic transitions in the origin of new evolutionary lineages (Hintz *et al.*, 2006). The present study documents several aspects of the *Nigella damascena* natural floral dimorphism in an attempt to understand the mechanisms underlying the success of a homeotic mutant in the wild and its evolutionary potential. We provide a quantitative analysis of differences in floral and reproductive traits between the two *Nigella damascena* floral morphs and a survey of pollinator behavior and visitation patterns in relation to the two morphs. Additionally we studied the dynamic of morph-ratio between two consecutive generations, which helped complete our understanding of reproduction modes. Finally we performed a regression analysis study to detect and estimate patterns of selection on quantitatively varying floral traits on both morphs.

Morph effect on pollinator behavior

Based on our previous knowledge on the *Nigella damascena* floral dimorphism morphology, we hypothesized that the striking differences observed between the two floral morphs would have an impact on pollinator behavior. Although we observed visitation by a wide variety of insects, we determined, based on frequency and specificity of behavior, that the potential effective pollinator assemblage of *Nigella damascena* in the geographical range of observation is composed by members of the *Apis* and *Bombus* genus. Effective pollinator assemblage is classically determined not only by the frequency of visitation, but perhaps more importantly by its efficiency, including the associated rates of pollen removal and deposition (Stebbins, 1970). Although we did not measure those rates

quantitatively, we were able to detect a qualitative difference in behavior patterns between insects in the *Apis* + *Bombus* group and others outside it. The specificity of these visitation patterns lies in a nectar-collecting focused behavior, in which insects approach a flower and proceed to 'pump' nectar from each petal doing a complete tour of the flower. We hypothesize that while such a behavior seems to effectively promote contact between the insect and the mature anthers and curved styles, it may also be responsible for a proportion of selfing by promoting contact between the different floral organs. Indeed, in other pollination systems, the presence of nectar has been associated with higher selfing rates, either through promotion of autogamy (within-flower pollination) or geitonogamy (between flowers of the same plant) (Jersáková & Johnson, 2006; Karron *et al.*, 2009). Additionally, because this visitation was not specifically observed at what is perceived as an optimal state for pollination (i.e. when it enables contact between pollen load on insect and receptive stigmatic surfaces, Toxopéus, 1927; Zhao *et al.*, 2011) but at all times throughout anthesis, it suggests that visitation may contribute independently to pollen removal and deposition.

The most striking result of the foraging behavior focused on nectar collection, is the strong correlation of visitation rates and floral morph. Indeed, petalous [P] morph flowers showed remarkably higher visitation rates and duration, whereas [T] morph flowers were almost always successfully avoided by pollinators. This apparent capacity of bees and bumblebees to distinguish the two floral morphologies from afar, could be the result of an associative learning process in which the insects perceive morph and associate it with the presence/absence of nectar, learning to prefer the most rewarding one (Schiestl & Johnson, 2013). In addition to a quantitative difference in visitation rate between the two morphs, a qualitative difference in effective pollen removal was also observed, as anthers of [P] morph flowers were significantly more depleted than those of [T] morph (personal observations during the open *vs.* hand pollination experimentation). Regardless of whether this pollen was mainly transferred within flowers, between flowers of a same plant or between plants, such differences between morphs, both in pollen removal and visitation rates, suggest that pollen transport occurs mainly among [P] plants.

Reproduction strategies

Since we confirmed the fundamental premise that pollinator behavior is affected by floral morph, we hypothesized that reproduction modes differ consequently between morphs. Since [P] morph flowers are frequently visited, we hypothesized that [P] plants do a substantial proportion of outcrossing with pollen transported by insects between flowers of [P] plants, with a potential minimal proportion of selfing, whereas the [T] morph, whose flowers are much less visited, depends exclusively on selfing for reproductive success. Because all [P] plants in the first generation (G_0) of our experimental population are heterozygous, both selfing and intra-[P] morph reproduction produce the same morph-ratios in progenies. Therefore we cannot directly discriminate the two modes but we can test, in a more general manner, a within-morph reproduction hypothesis. Surprisingly, we found an excess of [T] morph descendants, which led to the rejection of both a selfing and intra-[P] morph reproduction. Additionally, because [T] plants are homozygous, any departure from an exclusively selfing reproduction mode is easily detectable by direct observation of progeny morph. And indeed we found a non-negligible percentage of [P] morph plants in the [T] morph progeny, implying that [T] plants do a proportion of outcrossing, and this may be carried on with pollen from [P] plants. The proportion of [P] plants in the progeny of [T] plants gives an approximate indication of the rate of cross pollination of [T] morph plants by *P* pollen, which can only be issued from [P] plants. However, because G_0 [P] plants are heterozygous, they produce equal proportions *P* and *p* pollen, which, upon pollination of [T] morph flowers will lead to the production of both the easily detected [P] descendants, and [T] morph descendants which are indistinguishable from the descendents issued by selfing. Therefore, a better estimation of the rate of pollen flux from [P] plants to [T] plants should be the double of the proportion of [P] plants in the progeny of [T] plants. The real out-crossing rate of [T] plants, however, cannot be as easily estimated, as it involves not only pollination with pollen issued from [P] plants as discussed above, but also the possibility of pollination by pollen from other [T] plants. Because that pollen is recessive *p*, the result of pollination with it is phenotypically indistinguishable from pollination with *p* pollen from [P] plants or from selfing. We can assume, however that the rate of pollen flux between [T] plants is limited, based on our observations of a clear preference of pollinators

for [P] flowers. Alternatively, if pollinators do indeed develop a pollen-collecting behavior independent of morph, as it was seen in the second year of observations, that rate may increase. Be as it may, it is safe to assume that our appraisal of out-crossing rates grossly underestimate the total gene flux in the population. One way to improve on this estimation is to study the segregation patterns of different genetic markers in the progenies. In view of that, further genetic markers have been developed at the lab and plant material for DNA extraction was sampled from each descendant of the G_0 progenies.

The fact that insects visiting [P] plants can also on occasion visit [T] flowers, effectively depositing [P] pollen on their stigmas, suggests that the same insects can also transport pollen from a [T] plant to a [P] plant. However, since we observed a clear preference of pollinators for [P] flowers we have no reason to believe that the rates of pollen flux to and from [T] plants should be symmetrical. In fact, since [T] plants are rarely visited, transport of pollen from a [T] plant to a [P] plant is likely a rare event. Therefore, an unexpected pollen flux from [T] plants to [P] plants is likely not enough to explain the excess of [T] morph plants observed in the progeny of [P] morph plants. Surprisingly, despite having rejected the pollinator behavior hypothesis for [P] and [T] reproduction modes based on progeny results, the theoretical proportions issued from that hypothesis, which posits an intra-morph reproduction mode for [P] plants and a selfing mode for [T] plants, were suitably fitted to predict the following generation's morph-ratio (G_1) as observed in the field.

There was a surprising disparity between the expected 50-50 proportion of [P] and [T] plants and the observed morph-ratio in the initial generation (G_0). This distortion towards approximately 1/3 [P] and 2/3 [T] in all three experimental population replicates, suggests that there is a selective bias in favor of the [T] morph, which could be of pre-zygotic or post-zygotic nature. Although pre-zygotic bias are usually observed at the pollen-level, because all pollen used in the production of the G_0 was recessive *p*, selection at a pre-zygotic level is more likely to involve a mechanism favoring fecundation or production of *p* ovules, for example. Alternatively, a post-zygotic bias could involve a mechanism of selective abortion of heterozygous zygotes, selective canalization of resources to homozygous zygotes, or a combination of this and other mechanisms that confer the [T] morph an advantage in germination

and/or survival up to the flowering stage, in natural conditions. No significant germination rate differences were observed between the progenies of [P] and [T] plants. However, [P] morph progenies have a significant proportion of [T] plants, and progeny germination rate was tested in optimal artificial conditions. Therefore, those rates may not be good indicators of the real differential of germination potential between [P] and [T] morph seeds in natural conditions. Additionally, because the same bias was observed in all three population replicates, unless the selective advantage of the [T] morph is genetically conferred by a locus in close proximity to the *P* locus (see below), a differential selection mechanism after seed maturation is not very likely. An independent study of germination rate is currently underway at our lab in order to clarify the pre- or pos-zygotic origin of the morph-ratio bias, involving the germination in controlled optimal conditions of seeds issued from the same crosses that gave origin to the seeds used in the set-up of the experimental population, followed by individual genotyping of each seedling at the *P* locus.

As briefly mentioned above, a possible explanation for the selective advantage of [T] morph plants could be related to the weak genetic basis of the experimental population initial generation. Particularly, due to the small number of crosses that were performed to obtain the G_0 we can reasonably expect for a great number of loci to be in strong linkage disequilibrium in this population. Therefore, it is possible for the *P* locus to be in disequilibrium with a nearby locus affecting aspects of plant fitness, such as seed development, germination and/or plantlet vigor or survival. In such a scenario, selection could be explained by a linkage disequilibrium situation between the allele conferring an advantage and the recessive *p* allele, so that the homozygous combination present in [T] morph plants confers the higher fitness.

Interestingly, although a potential selective advantage of [T] morph plants is also suggested by the excess of [T] morph plants in the progeny of G_0 [P] plants, a scenario giving such an advantage to the [T] morph does not seem the most accurate in predicting the G_1 outcome in the field. In fact, as previously mentioned, the morph-ratio observed in the G_1 was closer to the 25.9 % [P] – 74.1 % [T] ratio expected under the initial pollinator behavior based hypothesis, than to the 18 % [P] – 82 % [T] proportion expected after taking into consideration the real outcomes of the progenies of G_0 [P] and

[T] plants which give an advantage to the [T] morph. Most likely, the proportion of [T] plants in the G_1 follows a mix of the two hypotheses. On the one hand both the G_0 and the progenies of [P] plants suggest an advantage of [T] plants, and on the other hand the high selfing rate of [T] morph plants could lead to an inbreeding depression in their offspring (see further below) which may partially overcome the advantage and offset the G_1 proportions.

Reproductive success

Since [P] and [T] flowers are visited and, most likely, pollinated differently, we might expect them to also have a different reproductive success. The immediate female reproductive success of a plant can be evaluated by the number of seeds produced, which integrates at once both a component of plant vigor and resource mobilization capacity and of fecundation potential. We tested for differences in capsule and seed production, and found that [T] morph plants produce bigger capsules but not a significantly greater number of capsules. Although the formation of capsules depends on successful fecundation of ovules, because self-pollination can occur in both morphs, and assuming that it provides the minimal fecundation necessary for fruit development, the number of capsules formed is more likely a reflection of the number of flowers, hence plant stature/vigor, than pollination efficiency.

While seed number is higher in [T] capsules, total seed mass per capsule does not differ significantly between the two morphs, suggesting that resource allocation for seed development is similar between the two morphs. However, while the number of seeds per capsule is greater in [T] plants, the mean weight per seed is higher in [P] plants, which suggests that reproduction strategies differ between the two morphs: [P] plants produce fewer but bigger seeds and [T] produce smaller but more numerous seeds. The difference in the number of seeds suggests that the number of fecundated ovules is higher in [T] flowers than in [P]. This difference could have a pollination efficiency related origin, or be due to an intrinsic difference in ovule production between the two morphs. If pollination efficiency is not limiting to seed production then differences in the number of ovules produced could be responsible for the difference in seed production, and we might expect to observe a smaller number of ovules in [P] carpels in a comparative assessment of the number of ovules produced by [P] and [T] morph.

Alternatively, if ovule number does not differ between the two morphs, the difference in seed number could be the result of a difference in pollination efficiency or a selective abortion of young zygotes. Pollination efficiency could be expected to yield differences in seed number if pollen deposition in [T] morph flowers is either greater or more effective. A study of the impact of pollen load on seed production has been set up via an open-pollination *vs.* hand-pollination with an excess of pollen experiment in the G_1 (see Material and methods section). Preliminary analysis of this experiment results showed no impact of pollen load on the duration of flower receptivity period. The study of its impact on seed production will be addressed in the future. Additionally, pollination mode could lead to a difference in seed number if the potentially higher outcrossing rate in [P] morph flowers favors the production of bigger seeds from ovules fecundated with allo-pollen in detriment of those produced by selfing, hence affecting at once seed size and number.

A secondary measure of reproductive success, which more closely approximates a global measure of fitness, is the germination potential and viability of a plant's progeny. The study of the progenies of G_0 [P] and [T] morph plants gave some indications that [P] plants descendants are more vigorous, notably, their production of a greater number of leaves. The absence of significant differences in the measures performed at later stages of progeny development could be due to the fact that these observations mix plants of different ages (as they were obtained simultaneously for the entire progeny set which displayed a wide range of germination dates), as opposed to the specific measures on 21-day-old plants. However, the inclusion of germination date as a covariate in the model testing for differences between the progenies of both morphs did not reveal any further differentiating characters (data not shown). Alternatively, because the number of leaves and height on 21-day-old plants from a progeny is positively correlated with its mean seed weight (data not shown), the greater number of leaves on [P] morph progenies could be a reflection of their better seed quality, i.e. the greater individual seed mass, of those seeds compared with [T] morph. Seed mass/quality could be expected to provide an initial advantage in earlier stages which would be diluted through time and no longer apparent in later stages.

Selection on floral traits

We used four different measures of relative fitness to determine whether floral traits on both morphs are subject to different selective pressures. Two of those measures are a direct transformation of the reproductive success traits discussed above: the total number of *Seeds* and the total *Seed mass* produced at the plant level. The other two fitness components are an integration of traits we believe to be related to overall plant fitness. The first (*PC1*) represents the relationship between capsule size and seed mass, and the second (*PC2*) represents the integration of plant vigor and progeny vigor. Only *PC2* is significantly different between morphs although the number of *Seeds* and the *Seed mass* also show a similar tendency, giving an advantage to [T] morph plants. *PC1*, despite not being significantly different between morphs, showed the inverse tendency. This is likely due to the strong contribution of mean seed weight to the elaboration of this trait which is greater in [P] plants. Most importantly, *PC1* and *PC2* represent only components of fitness, each incorporating a different set of variables. Therefore, careful ponderation of the two measures is required to obtain a global estimation of fitness, whereas the analysis of each one on its own may be useful in decomposing the total fitness in more relatable components.

The different fitness estimations revealed different modes of selection on flower diameter. *PC1* and *PC2* reveal opposing directional selection gradients on [P] morph flower diameter suggesting that selection at the capsule size and seed mass level drives flower diameter to bigger sizes, whereas selection at the plant stature and progeny viability level favors smaller flowers. Additionally, the *Seed mass* fitness component, which is the product of capsule number and seed number per capsule, each contributing to *PC2* and *PC1* respectively, revealed a positive quadratic selection gradient, suggestive of disruptive selection on floral diameter for small and big flowers.

Both the number of *Seeds* and the *Seed mass* suggest directional selection for a greater number of perianth organs in [P] flowers. The number of perianth organs in [P] plants is equally related to the number of sepals and petals, therefore, we cannot at this time advance any theories on whether this gradient reflects a pressure to increase attractiveness or reward. *PC2* revealed an asymmetrical

stabilizing selection gradient on the number of perianth organs of [T] flowers suggesting that increasing the number of sepal-like organs in this morph is only favorable to a certain point.

Major conclusions and perspectives

Evidence for selection on floral traits is ambiguous, particularly at the flower diameter level. While having bigger flowers may seem advantageous from a reproductive fitness point of view, better fitness at the level of plant and progeny vigor is associated with smaller flowers. Additionally, increasing the number of perianth organs seems to be advantageous in [P] plants but not in the [T] morph to which there seems to exist a limiting optimum. The number of perianth organs, on the other hand, has a coherent optimum across fitness measures suggesting an advantage for plants producing a greater number of organs. Regardless of the gradients revealed by the different fitness measures, for selection on floral traits to result in changes in floral trait distribution across generations the observed phenotypic variability needs to have a heritable genetic base (Brodie III *et al.*, 1995). Although we did find an effect of mother plant on progeny value suggesting a genetic origin of phenotypic variability for the traits of progeny viability, our experimental set-up did not allow us to test the genetic basis of floral trait variance. A future study of floral traits variability within and between families and the corresponding estimation of the different traits heritabilities will be required in order to understand the potential effects of the observed selective gradients on the evolution of floral traits distribution. On the other hand, the floral morph seems to have an effect on the strength and optima of selection on individual traits. Thus, in order to understand floral trait evolution the population genetic and morph structure needs to be taken into account. Namely, if selection gradients on a floral trait have opposite signs on the two morphs, a polymorphic situation regarding floral morph may prevent the population from reaching the optimum for each individual floral trait. Otherwise, if the two morphs remain isolated in monomorphic populations, such as [P] plants in natural conditions and [T] morph plants in cultivation, traits may evolve independently in different directions.

We found surprising evidence for an advantage of apetalous [T] morph plants in natural conditions, when compared to the petalous wild-type morph [P] morph. As suggested above, the origin of this

advantage could lie in the narrow genetic structure of our plant material, and an association between the *P* locus and a closely linked locus with effects on plant vigor and viability. Because an eleated proportion of the *p* alleles responsible for the [T] morph originated from commercial seed lots, we hypothesize that the association between this allele and the allele conferring higher vigor and viability could have been produced by a history of artificial selection for bigger and fitter plants in the horticultural medium. Conversely, the genetic background of the *P* allele in natural populations likely harbors greater variability. In view of studying this variability, a survey and sampling of several natural populations in the south of France has been conducted by lab members. In spite of this advantage of [T] plants, which is visible both in the G_0 and its progeny, there is also evidence for an inbreeding depression in the progeny of [T] plants as a result of their mostly selfing reproduction mode. Hence, understanding the evolution of these polymorphic populations requires an appreciation of the balance between these two factors. Our observations suggest that the inbreeding depression associated with the reproduction mode of [T] plants can compensate to some extent the advantage associated with the genetic background of [T] morph plants, and shift the morph-ratio towards a more balanced scenario. However, during the course of this study only two generations were observed which limits the scope of our hypothesis on the outcome of a polymorphic situation. As a result, the continued study of morph-ratio evolution across several generations, as well as continued comparative studies of reproduction modes and fitness evolution in both morphs will be required to discern solid evolutionary trends of natural *Nigella damascena* populations in a polymorphic state.

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Supporting information

Supplementary table 1. Progeny traits, means ± SD. *F* (residual degrees of freedom): statistics for morph comparison, from a model considering the effects of morph, population replicate and the interaction between the two. Means in bold correspond to the higher value when significant. * *P* < 0.05, ** *P* < 0.01, * P < 0.001

	N	Number of leaves at 21 d.a.g.	Plant height at 21 d.a.g. (cm)	Number leaves at 23 d.a.o.g.	Plant height at 23 d.a.o.g. (cm)	Plant height at 42 d.a.o.g. (cm)	Difference of height between the 42nd and 23rd d.a.o.g. (cm)
		Comparison of progeny traits by morph of descendants					
[P]	365	**5.93 ± 1.23**	113.85 ± 19.96	5.55 ± 1.82	107.13 ± 20.65	203.44 ± 72.89	106.81 ± 63.65
[T]	1017	5.65 ± 1.12	112.19 ± 19.69	5.26 ± 1.76	106.10 ± 22.75	205.12 ± 76.03	106.93 ± 66.06
F	(r.d.f.)	9.01 (1331) **	2.04 (1330)	2.34 (1246)	0.16 (1246)	0.63 (1369)	0.20 (1246)
		Comparison of progeny traits between [P] and [T] descendants of [P] mothers					
[P]	352	5.95 ± 1.31	115 ± 21.4	5.53 ± 2.01	106 ± 21.8	200 ± 74.2	105 ± 63.3
[T]	365	5.91 ± 1.15	113 ± 18.4	5.57 ± 1.62	108 ± 19.6	207 ± 71.5	109 ± 64.0
F	(r.d.f.)	0.50 (687)	0.44 (687)	0.09 (632)	1.26 (632)	1.01 (707)	0.001 (632)
		Comparison of progeny traits between [P] and [T] descendants of [T] mothers					
[P]	13	5.75 ± 0.97	117 ± 17.6	5.73 ± 2.20	107 ± 25.5	226 ± 90.2	**136 ± 61.7**
[T]	652	5.65 ± 1.12	112 ± 19.7	5.25 ± 1.75	106 ± 22.7	205 ± 75.8	106 ± 66.1
F	(r.d.f.)	0.38 (636)	0.39 (635)	1.38 (606)	0.014 (606)	2.62 (654)	4.87 (606) *
		Comparison of progeny traits of [T] morph descendants of [P] and [T] mothers					
[P]	365	**5.91 ± 1.15**	113 ± 18.4	**5.57 ± 1.62**	108 ± 19.6	207 ± 71.5	109 ± 64.0
[T]	652	5.65 ± 1.12	112 ± 19.7	5.25 ± 1.75	106 ± 22.7	205 ± 75.8	106 ± 66.1
F	(r.d.f.)	11.90 (978) ***	1.08 (977)	6.57 (925) *	1.71 (925)	0.012 (1005)	0.020 (925)
		Comparison of progeny traits of [P] morph descendants of [P] and [T] mothers					
[P]	352	5.95 ± 1.31	115 ± 21.4	5.53 ± 2.01	106 ± 21.8	200 ± 74.2	105 ± 63.3
[T]	13	5.75 ± 0.97	117 ± 17.6	5.73 ± 2.20	107 ± 25.5	226 ± 90.2	**136 ± 61.7**
F	(r.d.f.)	0.16 (345)	0.07 (345)	0.13 (313)	0.002 (313)	2.65 (356)	4.53 (313) *

Supplementary table 2. Relative contributions of different vigor and reproduction associated variables on the two first principal components of a PCA, and the Pearson's product moment correlation coefficients (r2) for each variable and the two components.

	PC1	r^2	PC1	r^2
Plant height	0.04	0.047	17.88	0.677
Capsule number	3.22	0.401	13.02	0.578
Capsule length	12.75	0.781	7.28	0.428
Capsule diameter	12.43	0.771	2.86	0.268
Seeds per capsule	8.07	0.621	11.11	0.528
Seed mass per capsule	14.00	0.819	3.18	0.283
Mean seed weight	12.74	0.781	0.95	-0.154
Number of leaves j21	7.84	0.613	2.84	-0.267
Plant height j21	3.98	0.437	12.14	-0.552
Number of leaves j23	9.60	0.678	11.06	-0.527
Plant height j23	8.06	0.621	15.00	-0.614
Plant height j42	6.28	0.548	1.94	-0.221
Germination rate	0.96	0.214	0.75	0.137
Percentage of variance explained	36.83 %	4.79	19.31 %	2.51

Conclusion

“Knowing where you came from is no less important than knowing where you are going.”

— Neil deGrasse Tyson

Brève résumé en français de la conclusion

Bien que ne montrant pas le classique scénario de transformation homéotique, ou peut-être à cause de cela, le dimorphisme floral *Nigella damascena* constitue un cas remarquable d'origine d'une nouveauté évolutive. Notre étude de ses aspects écologiques nous a fourni quelques idées sur les limitations qui accompagnent ces transformations morphologiques spectaculaires, notamment un changement potentiel de mode de reproduction entre pollinisation croisée et autofécondation. Toutefois, les singularités de ce polymorphisme, à savoir la reproduction artificielle continue de la forme mutante par l'homme et son potentiel de reproduction par autofécondation, sont clés pour la compréhension de son potentiel évolutif. Les principaux résultats de ce domaine de la thèse sont discutés ainsi que de nouvelles perspectives pour la recherche de ce modèle.

Loose ends

Not a monster and not a homeotic mutant but still hopeful

During the study of the *Nigella damascena* floral dimorphism and the compared analysis of floral morphologies described in the Chapter 1, we observed a greater total number of perianth organs in the flowers of the apetalous [T] morph, when compared with the [P] morph flowers. We hypothesized that this could not be achieved by simple transformation of petals into sepal-like organs, but that a greater number of organ primordia had to be formed, excluding the possibility of a simple homeotic transformation mechanism. In the course of the *in situ* study described in Chapter 2, we confirmed that a superior number of total perianth organs is indeed produced in [T] morph flowers. Moreover, by studying the number of each type of organs, we verified that this is in fact caused by an augmentation of the total number of formed primordia. Indeed, despite having fewer stamens, the apetalous flowers of the [T] morph have a greater number of floral organs in total, reinforcing our initial suggestion of an increase in perianth organ number via a disruption of the developmental program controlling organ primordia formation.

Despite not fitting the classical homeotic transformation scenario, or perhaps in virtue of it, the *Nigella damascena* floral dimorphism constitutes a remarkable case of evolutionary novelty origin. Our study of its ecological aspects in natural conditions has provided us with some ideas of the limitations that accompany such dramatic morphological transformations, i.e. a potential shift from an outcrossing to a selfing pollination mode. However, the singularities of this polymorphism, namely the continuous artificial breeding of the mutant form by man and its potential to reproduce via a selfing pollination mode, may just be the key to its evolutionary potential.

The journey of a mutant

One aspect that was not approached by this thesis but is nonetheless of great interest is the origin, history and outcome of the apetalous mutant. A study based on the sampling of different geographical accessions (namely across the original area of distribution of the species) of both petalous and mutant forms, and the characterization of their *P* and *p* alleles could set the basis for a phylogenetic study and reconstruction of the history of this form. Notably, it would be interesting to confirm the derived nature and single origin of the mutant allele responsible for the apetalous form and its geographical foundation, as well as the history of its "domestication" by man and dissemination around the world as a horticultural variety. As the [T] form grows in popularity as a horticultural variant, we could hypothesize that the transference of this morph to natural populations will become more frequent, making it relevant to assess its prevalence both in artificial and natural conditions and understanding the mechanisms of its propagation. By studying the two forms in an artificially created polymorphic population grown in experimental semi-natural conditions we learned a few aspects about this dimorphism, which may inform us of the outcome of a polymorphic situation in natural populations. However, some of these aspects still need further confirmation.

Notably, although we found a potential advantage associated with the recessive *p* allele responsible for the apetalous phenotype which could suggest that this morph may be favorably selected, it is not clear whether this advantage is a general phenomenon or if it is specific of our material. The narrow genetic basis of our G_0 population, in particular, could have produced a linkage disequilibrium situation between the *P* locus and a fitness locus nearby. This issue could be addressed by crossing [T] plants with [P] plants from different genetic backgrounds, including the different natural populations sampled in the south of France during our survey, and studying the fitness of their progenies.

In addition we also showed that, due to the mostly selfing reproduction mode of [T] plants, their progenies may suffer from a lower fitness resulting from inbreeding depression which does not seem to occur in the descendants of [P] morph plants. These results should be confirmed by

estimating in a more precise way the outcrossing and selfing rates of the two morphs using genetic markers. Additionally, quantifying inbreeding depression levels on [P] plants from populations compared with [T] plants from horticultural origin could inform us on whether the [T] morph has reduced its genetic load by purging deleterious alleles. If there is genetic purging in the artificially cultivated [T] populations we could expect this morph to suffer from less inbreeding depression when reproducing via selfing in natural populations, having an advantage when compared to [P] plants in small inbred populations. On the other hand, outcrossing with [P] plants may limit this advantage by restoring their levels of allelic diversity, including alleles that may have unfavorable effects. Indeed, the signs of inbreeding depression observed in the [T] progenies suggest that, by crossing artificially bred [T] plants with [P] plants from natural populations to produce the G_0 of our experimental population, we exposed the former to an important genetic load which became unfavorable upon selfing. This scenario is clearly different from an event of migration of [T] plants to natural populations which we could hypothesize to be accompanied by lower inbreeding depression levels, at least during the initial generations after introduction.

Put together, the reduced inbreeding depression of [T] plants due to purging and the potential selective advantage due to a favorable allele linked to the *NdAP3-3 p* allele suggests that [T] plants that migrate from an artificial population to a natural [P] morph population may not be all that "doomed" and could be maintained for at least several generations.

In addition to the considerations on the genetic effects of selfing, the potential advantage provided by the ability for the species to assure reproduction independently of pollinator visitations should also be considered. Indeed, [T] plants that have purged their deleterious alleles could have a crucial advantage in the absence of pollinators. This advantage, and in a more general way, the reduced genetic load of [T] morph plants, may allow it to persist in natural conditions until a new pollination niche is found. Interestingly, and along that line of thought, between the first and second generations we observed some variation in pollinator behavior, including a transition between nectar-collecting foraging patterns, which concerned

only the petalous form, and pollen-collecting behavior concerning both morphs. The prevalence of these behaviors is likely influenced by environmental factors, the composition of the flowering plants community and other aspects of pollinator ecology and physiology, highlighting the importance of external factors to the rate of outcrossing within a population or within a certain form in a population.

Therefore, it will be equally interesting to study the evolution of the reproduction mode of this morph, in addition to its relative fitness compared to the wild-type morph, in order to determine its potential to form an independent lineage and to conjecture evolutionary scenarios. Such as whether the [T] morph can overcome the [P] morph in a competitive scenario where both morphs assure their reproduction ([P] by outcrossing and [T] by selfing and reducing the genetic load), whether the two morphs can both be maintained and evolve independently eventually becoming reproductively isolated, or whether a minimal proportion of inter-morph crossing will prevent the [T] morph from keeping its potential advantage for a long period and eventually be lost due to a lower fitness.

A box of surprises

In the second year of observations (G_1) we noticed an alternative phenotype to the previously described petalous [P] and apetalous [T] morphs, which also affected the perianth organs (**Figure 1**, Top). This phenotype consisted in the production of petals with sepal characteristics at their apex, ranging from light coloration to the elongation of the two lobes into blade like structures resembling the sepal blade. This phenotype differed from the incomplete transformation of petals that we observed in the VIGS experiment (Chapter 1). The production of these organs varied in number and was occasionally accompanied by the production of a variable number of supernumerary perianth organs. However, these forms were seen in all three replicates of our experimental population in a structured fashion across the plots (that is, variant plants were observed in patches suggesting a potential organization by families), as well as among the progeny sown in greenhouse. These observations, along with the fact that all flowers

of an "affected" plant showed the alternative phenotype, albeit to varying degrees, highly suggest that the formation of such organs has a genetic basis.

Figure 1. Alternative floral phenotypes observed in the second generation of the experimental population. Top: alternative [P] morph with petals showing different degrees of sepal-like identity (white arrowheads), notably elongation of the two lobes into sepal blades and sepal-like light coloration. Bottom: alternative [T] morph showing aberrantly formed carpels in the position of stamens (black arrowheads).

The occurrence of this alternative form in the second generation could suggest that the two disruptions of perianth architecture observed in the apetalous form (petal loss and organ number and boundary deregulation) correspond to two discrete but closely linked functions that can become decoupled by recombination. However, whether these functions are performed by different functional or regulatory regions of the single locus *P* or by two very closely related loci, it should not affect our previous results and interpretations on the role of *NdAP3-3* on the *Nigella damascena* flower development, which are well substantiated by the phenotypical results of the silencing experiments. Indeed, one explanation for why the alternative form was not observed earlier could be that the two phenotypes are dominant and caused by two linked genes. Assessing the frequency of this new form could give an estimation of the recombination rate between the two loci. Seeds from a series of plants showing the new phenotype were collected which can be used in a genetic study to determine its genetic basis, namely the determination of number and the relationship between the implicated loci via segregation analysis of different crosses. Alternatively, the new phenotype could result from a change in the interaction between NdAP3-3 and its co-factors such as PI and SEP or from a differential

regulation of its activity, both of which could depend on nearby sequences and an interaction with the genetic background. Interestingly, the fact that this new phenotype affects differently the apex and base of petals suggests a connection with the late expression pattern of *NdAP3-3* which appears to become restricted to the apex of developing petal primordia in later stages (Chapter 1). This specific expression dynamic could be related to a role in the specification of mature petal characteristics, which could be disrupted in the alternative form by a deregulation of the late spatial restriction of the *NdAP3-3* expression. Such a deregulation could result from an alteration of cis-regulatory mechanisms of *NdAP3-3* expression or from an alteration in epigenetic marking at a nearby sequence, possibly taking place during petal development. The latter is all the more plausible that the degree of transformation of petal lobes in sepal-like blade may vary in a single flower. Therefore, in addition to the study of NdAP3-3 co-factors and targets in a compared fashion between the two floral morphs, and the study of the potential mechanisms of its inactivation in the apetalous form (see discussion of Chapter 1) it could be interesting to study in detail the expression pattern dynamic of *NDAP3-3* during later stages of petal development and investigating the mechanisms of its regulation.

In addition to the "sepaloid" petals described above, another aberrant phenotype was also observed in a recurrent fashion in natural conditions, consisting in the production of malformed carpels in the third whorl of apetalous [T] morph plants, suggesting a potential shift in the C-function domain (**Figure 1**, Bottom). The occurrence of such organs in the mutant phenotype further enhances the idea of a major disruption of regulatory mechanisms during the floral meristem patterning and organ development processes in the mutant form, which adds to the interest of studying the network of genes affected by the presence/absence of *NdAP3-3* expression.

Résumé – Origine génétique et moléculaire, et rôle adaptatif d'un dimorphisme floral chez **Nigella damascena** *L.*

Comprendre la diversité morphologique des fleurs passe par l'étude de son origine moléculaire et développementale et de ses conséquences fonctionnelles et écologiques. Le périanthe est composé d'organes stériles, sépales et pétales, qui jouent un rôle majeur dans le succès reproducteur des plantes pollinisées par les animaux du fait de leur fonction d'attraction.

Cette thèse propose une approche multidisciplinaire visant à comprendre l'origine génétique et moléculaire de la diversité morphologique du périanthe et sa signification évolutive, à l'aide du modèle *Nigella damascena* L. Cette Renonculacée présente un dimorphisme spontané. La forme probablement ancestrale, trouvée en populations naturelles, a un périanthe bipartite composé de cinq sépales pétaloïdes et huit pétales nectarifères. Dans la forme variante, cultivée à des fins d'horticulture, les pétales sont remplacés par un nombre élevé d'organes allant d'une forme proche des sépales à une forme proche des étamines.

La première partie de cette thèse est consacrée à l'étude de l'origine développementale, génétique et moléculaire du dimorphisme, par la caractérisation détaillée de la morphologie florale et de son développement dans les deux morphes dans le cadre d'une approche gène candidat. Par analyse d'expression et validation fonctionnelle, nous avons montré que le gène *NdAP3-3* est responsable de l'ensemble des aspects du dimorphisme floral de *N. damascena*, ce qui suggère que ce gène joue un rôle dans l'identité du pétale mais aussi dans l'architecture du méristème, potentiellement via la régulation du nombre d'organes et de la frontière entre périanthe et étamines.

La seconde partie de cette thèse concerne l'impact du dimorphisme floral sur le mode de reproduction des deux morphes et leur maintien potentiel. Nous avons caractérisé les stratégies reproductives et la valeur sélective des deux morphes en conditions naturelles dans des populations expérimentales. Le variant sans pétale est peu visité par les pollinisateurs, et se reproduit majoritairement en autogamie. L'analyse de la vigueur de ses descendants suggère une dépression de consanguinité. Par ailleurs, dans notre matériel, il semble que l'allèle donnant le phénotype sans pétale soit lié à un allèle augmentant la valeur sélective. A la lumière de nos résultats, nous discutons les conditions du maintien de ce polymorphisme.

Mots-clés:
Ranunculaceae, *Nigella damascena*, dimorphisme floral
Développement floral, architecture du périanthe, identité pétale, gènes de fonction B, *APETALA3-3*
Comportement des pollinisateurs, valeur sélective, système de reproduction, sélection sur caractères floraux

Abstract – A floral dimorphism in **Nigella damascena** *L.: genetic and molecular control, and adaptive significance*

Understanding flower diversity requires on one hand the study of the molecular and developmental origin of floral architecture, and on the other the study of the functional and ecological consequences of flower morphology. A great deal of that diversity can be found at the perianth level which comprises the sepals and petals, sterile and versatile organs that play a major role in the reproductive success of animal pollinated flowering plants through their attractive characteristics.

This thesis is the result of a multidisciplinary effort to understand the genetic and molecular origin as well as the evolutionary significance of perianth diversity, using the *Nigella damascena* L. as a model. This Ranunculaceae species presents a rare naturally occurring floral dimorphism affecting perianth architecture. The putatively ancestral form found in natural populations has a well differentiated bipartite perianth composed of five petaloid sepals and eight nectariferous petals, while the perianth in the alternative apetalous mutant, cultivated for horticultural purpose, has no petals and but is instead composed of numerous organs showing a continuum of forms from outer sepal-like to inner stamen-like.

The first part of this thesis was dedicated to the study of the developmental, genetic and molecular origin of this dimorphism, via a detailed characterization of floral morphology and development in both morphs, which laid a foundation for the interpretation of the results of a candidate gene approach. Using expression analysis and functional validation we showed that *NdAP3-3* is fully responsible for the complex *N. damascena* floral dimorphism, suggesting that it plays a role not only in petal identity but also in meristem patterning, possibly through the regulation of perianth organ number and perianth-stamen boundary.

The second half of this thesis focused on the impact of the floral dimorphism on the reproduction mode and evolutionary maintenance of the two morphs. We assessed reproduction strategies and reproductive success in the two morphs by studying a polymorphic experimental population in natural conditions. The absence of petals in the mutant form was associated with a qualitative drop in pollinator visitation which resulted in a shift towards selfing. The study of their progeny suggests that selfing had a negative effect on the descendant's vigor via inbreeding depression. Additionally, in our material, the allele responsible for the apetalous phenotype seems to be linked to a favorable allele increasing fitness. We discuss the mechanisms of the dimorphism maintenance in light of these results.

Keywords:
Ranunculaceae, *Nigella damascena*, floral dimorphism
Flower development, perianth architecture, petal identity, B-function genes, *APETALA3-3*
Pollinator behavior, fitness, mating system, selection on floral traits