

Génétique de la floraison de plantes d'intérêt agricole et horticole.

Fabrice Foucher

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MEMOIRE

Présenté devant l'Université d'Angers En vue de l'obtentiont de

L'Habilitation à Diriger des Recherches

par

Fabrice FOUCHER

Chargé de Recherche INRA UMR Institut de Recherche en Horticulture et Semences (INRA/ Agrocampus Ouest / Université d'Angers)

Génétique de la floraison de plantes d'intérêt agricole et horticole.

Soutenue publiquement le 10 décembre 2012 devant le jury composé de

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Examinateurs :

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Un grand remerciement à Catherine Rameau pour les 2 années exceptionnelles passées à travailler sur le pois et sur la floraison à Versailles. Enfin un grand merci aux personnes d'Angers pour m'avoir accueilli si chaleuresement. Mes remerciements vont plus particulièrement à Laurence et à Elisabeth. Vous avez joué un rôle important dans la réussite du projet rosier sur Angers.

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1. Titre et résumé des travaux.

Génétique de la floraison de plantes d'intérêt agronomique et horticole.

Après une thèse sur le développement de la luzerne (*M. truncatula*), mes travaux de recherche ont principalement porté sur le contrôle génétique et moléculaire de la floraison chez une plante annuelle d'intérêt agronomique (le pois) puis chez une plante pérenne ornementale (le rosier). L'objectif était d'isoler des gènes clés du contrôle de l'initiation florale et de comprendre leur régulation et leur diversité avec comme enjeu le contrôle de la floraison (précocité de la floraison du pois d'hiver et floraison du rosier). Pour cela, une approche gènecandidat a été développée en comparant les données moléculaires disponibles chez la plante modèle annuelle Arabidopsis thaliana et les données physiologiques disponibles chez les plantes d'intérêt. J'ai ainsi montré le rôle important joué par les répresseurs floraux de la famille TFL1 (TERMINAL FLOWER 1). Chez le pois, un homologue de TFL1, LATE FLOWERING, contrôle la précocité de floraison alors qu'un autre homologue, DETERMINATE, est impliqué dans l'architecture de l'inflorescence. Chez le rosier, KSN, un homologue de TFL1, est un régulateur de la floraison continue du rosier. Nous avons montré qu'il était régulé par les gibbérellines. Ces résultats originaux sur le contrôle de la floraison chez une plante pérenne ont été poursuivis par des approches de génétique fonctionnelle, de physiologie moléculaire et d'analyse de diversité. Ces études ont permis de mettre en évidence la conservation des gènes contrôlant la floraison entre différentes plantes présentant des modes de floraison très différents (monocarpique versus polycarpique). Ces plantes ont développé différentes stratégies en termes de régulation des gènes (duplication et spécialisation chez le pois, régulation par les GAs chez le rosier).

2. Curriculum vitae

FOUCHER Fabrice

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EXPERIECES PROFESSIONNELLES

- Depuis 2012 : Au sein de l'Institut de Recherche en Horticulture et Semences (IRHS ; INRA, Agrocampus Ouest, Université d'Angers), je co-anime avec Agnès Grapin (Enseignant-Chercheur à Agrocampus Ouest) l'équipe « Déterminisme génétique et diversité des plantes ornementales » (GDO). Au sein de cette équipe, je développe une thématique sur les bases génétiques et moléculaires de l'architecture du rosier. Au sein d'un consortium international, je m'implique dans le séquençage du génome du rosier.
- <u>2003-2011</u> : A l'UMR Génétique et Horticulture (GenHort, INRA/INH/ Université, Angers), j'ai animé un projet sur l'architecture du rosier. La thématique a été centrée sur le déterminisme génétique de la floraison et plus particulièrement de la remontée de floraison. Nous avons étudié le déterminisme génétique de caractères ornementaux importants (floraison, architecture, parfum). Nous avons isolé et caractérisé le gène de la remontée de floraison (homologue de *TFL1*) chez le rosier, et montré que ce dernier est régulé par les gibbérellines. Par une approche génétique, nous avons montré un rôle plus global des gènes de la famille *TFL1* dans l'architecture du rosier.
- <u>2000-2002</u>: Dans le cadre d'un projet Génoplante (GOP PEA A-G) dans l'équipe de Catherine Rameau (Station de Génétique, INRA, Versailles), j'ai étudié le contrôle de l'initiation florale chez le pois par une approche gène-candidat. Nous avons isolé et cartographié 15 nouveaux gènes. Cette étude a permis de caractériser 2 gènes similaires (homologue au gène *TFL1* d'*Arabidopsis*) impliqués dans l'initiation et le développement floral. De plus, en collaboration avec une équipe australienne (Jim Weller, Hobart), nous avons pu réaliser une analyse comparative du contrôle de la floraison entre les Fabacées (Pois, *Medicago truncatula*) et *Arabidopsis*.
- <u>1995-1999</u>: Au cours de ma thèse, au sein d'une équipe internationale étudiant le cycle cellulaire chez les végétaux (laboratoire d'Eva Kondorosi, ISV, CNRS, Gif/Yvette), j'ai analysé la fonction d'un gène lié à la division cellulaire, cycline de type D3. Nous avons montré que ce gène est impliqué dans la réactivation du cycle cellulaire et nous avons proposé un nouveau rôle de ce gène lors de la différentiation cellulaire chez Medicago.
- <u>Fév 95 Avril 95</u> : Dans le cadre de mon DEA, en collaboration avec 2 chercheurs (ISV, CNRS), j'ai étudié la stabilité d'un facteur bactérien (facteur Nod) vis à vis

d'enzymes végétales par une approche biochimique, et montré la dégradation spécifique de ce facteur par une hydrolase racinaire.

<u>Eté 1994</u>: Lors d'un stage (3 mois) dans un laboratoire dirigé par M. Hahn (Complex Carbohydrate Research Center, Athens, USA), j'ai apporté ma contribution à l'étude de la transduction d'un signal par une approche pharmacologique chez le soja.

ETUDES ET DIPLOMES

<u>1996-99</u> : Doctorat en Pathologie Végétale de l'Institut National Agronomique Paris-Grignon, Mention très honorable avec les félicitations du jury

<u>1992-95</u> : Ecole d'Ingénieurs (Institut National Agronomique Paris-Grignon)

Diplôme d'Ingénieur Agronome de l'INA P-G

Diplôme d'Etudes Approfondies spécialité Phytopathologie, Mention Bien

DOMAINES DE COMPETENCE

Scientifique : Conception, mise en œuvre et réalisation de programme de recherche en génétique quantitative et fonctionnelle sur le développement des plantes d'intérêt.

Technique : Biologie moléculaire, expression des gènes, développement marqueurs génétiques, transcriptomique (macroarray, puce Affymétrix®, RNA Seq)

Informatique : Bio-analyse (Vector NTI, Genious, ChromaPro), analyse phylogénétique **Management** :, Animation d'équipe, Encadrement (Techniciens, étudiants du L3 au postdoctorant), Organisation congrès (Nationaux et internationaux)

FORMATIONS SUIVIES

* Classification botanique et phylogénie (3 heures, 2011)

* Management animation d'équipe (2 jours, 2011)

* Accompagnement projet fusion GenHort-SAGAH (2 jours, 2009)

* Savoir répondre au média (2 jours, 2008)

* Réflexives® Linguistique et Pratiques de Recherche, Séminaire Encadrants - Doctorants, Fréjus (5 jours, Mai 2007)

* Expression des génomes (2006): du Northern à la puce (3h), Transcriptomique (6h) et Q-PCR (3h)

* Ecole Chercheur : 4^{ème} Ecole thématique de Biologie Végétale « Biologie Intégrative », Batz sur Mer (5 jours, Octobre 2005)

* Formation à la plate-forme ALEA (Atelier Logiciel libre pour l'étude et la simulation de modèles écophysiologiques), Montpellier (5 jours, 2004)

* Formation bio-informatique : Introduction à l'analyse phylogénétique (2 jours, 2004)

* Conduite de l'entretien d'évaluation, Angers (1 jour, 2004)

* Gestion de projet (2 jours, 2003)

* Technique d'analyse de la variabilité génétique (1 journée, 2003)

RESPONSABILITES COLLECTIVES

* Elu au conseil scientifique du département Génétique et Amélioration des Plantes (INRA, depuis 2011)

* Membre de la Commission Scientifique Spécialisée (CSS) « Ecophysiologie, Génétique et Biologie Intégrative des Plantes » (EGBIP, depuis 2012).

* Membre de la Commission Recherche de la CGT (depuis 2011).

* Animateur du groupe qualité biologie moléculaire (UMR GenHort Angers)

* Animateur du comité éditorial de l'UMR (2007-2011)

* Elu au Conseil de Service de l'UMR (2004-2009)

* Membre du comité éditorial de la revue du centre INRA Angers-Nantes (Ad Litteram, 2008-2011)

* Animateur du collectif local de « Sauvons la recherche » et co-président de l'association « Sauvons la recherche Pays de la Loire » (2003-2008).

ENSEIGNEMENT

* Co-animateur d'un module d'une semaine (Génétique du développement) dans le M2 Recherche « Biologie Intégrative » de l'Université d'Angers (2005-2008)

* Enseignement à Agrocampus Ouest au niveau M1 (3h, Physiologie de la floraison (2008-2012); 3h Génétique du rosier (2006-2009)

* Enseignement dans le module « Développement » du M2R BioVigPa (3h, Contrôle moléculaire de la floraison (2009-2012)

3. Liste des titres et travaux.

1. Articles scientifiques

1.1 Articles publiés (revues avec comité de lecture)

- Randoux, M., Jeauffre, J., Thouroude, T., Vasseur, F., Hamama, L., Juchaux, M., Sakr, S. and Foucher, F. (in press) Gibberellins regulate the transcription of the continuous flowering regulator, RoKSN, a rose TFL1 homologue. *J Exp Bot*. (IF 5.3, cited 0).
- Hamama, L., Naouar, A., Gala, R., Voisine, L., Pierre, S., Jeauffre, J., Cesbron, D., Leplat, F., Foucher, F, N. Dorion and Hibrand-Saint Oyant L. (in press) Overexpression of RoDELLA impacts the height, branching, and flowering behaviour of *Pelargonium x domesticum* transgenic plants. *Plant Cell Reports* (IF 2.2, cited 0).
- Iwata, H, Gaston, A, Remay, A, Thouroude, T, Jeauffre, J, Kawamura, K, Hibrand Saint-Oyant, L, Araki, T, Denoyes, B, and Foucher, F. 2012. The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. The Plant Journal 69, 116-125 (IF 6.16, cited 3)
- Choubane, D., Rabot, A., Mortreau, E., Legourrierec, J., Peron, T., **Foucher, F.,** Ahcene, Y., Pelleschi-Travier, S., Leduc, N., Hamama, L. and Sakr, S. (2012) Photocontrol of bud burst involves gibberellin biosynthesis in Rosa sp. *Journal of Plant Physiology*, online (IF 2.79, cited 0).
- Dubois, A., Remay, A., Raymond, O., Balzergue, S., Chauvet, A., Maene, M., Pécrix, Y., Yang, S.-H., Jeauffre, J., Thouroude, T., Boltz, V., Martin-Magniette, M.-L., Janczarski, S., Legeai, F., Renou, J.-P., Vergne, P., Le Bris, M., Foucher, F. and Bendahmane, M. (2011) Genomic Approach to Study Floral Development Genes in *Rosa sp. PLoS ONE*, 6, e28455. (IF 4.1, cited 0)
- Spiller, M., Linde, M., Hibrand-Saint Oyant, L., Tsai, C.-J., Byrne, D., Smulders, M., Foucher, F. and Debener, T. (2011) Towards a unified genetic map for diploid roses. Theor Appl Genet, 122, 489-500 (IF 3.4, cited 7)
- Kawamura, K., Hibrand-Saint Oyant, L., Crespel, L., Thouroude, T., Lalanne, D. and Foucher, F. (2011) Quantitative trait loci for flowering time and inflorescence architecture in rose. Theor Appl Genet, 122, 661-675 (IF 3.4, cited 4)
- Remay, A., Lalanne, D., Thouroude, T., Le Couviour, F., Hibrand-Saint Oyant, L., and Foucher, F. (2009). A survey of flowering genes reveals the role of gibberellins in floral control in rose. Theor Appl Genet 119, 767-781 (IF 3.4; cited 12)
- Lejeune-Hénaut, I., Hanocq, E., Béthencourt, L., Fontaine, V., Delbreil, B., Morin, J., Petit, A., Devaux, R., Boilleau, M., Stempniak, J.J., Thomas, M., Lainé, A.L., Foucher, F., Baranger, A., Burstin, J., Rameau, C., and Giauffret, C. (2008). The flowering locus Hr colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum* L. Theor Appl Genet **116**, 1105-1116. (IF 3.4, cited 13)
- Hibrand-Saint Oyant, L., Crespel, L., Rajapakse, S., Zhang, L., and Foucher, F. (2008). Genetic linkage maps of rose constructed with new microsatellite markers and locating QTL controlling flowering traits. Tree Genet. Genomes 4, 11-23. (IF 2.4, cited 6)
- Foucher, F., Chevalier, M., Corre, C., Soufflet-Freslon, V., Legeai, F., and Hibrand-Saint Oyant, L. (2008). New resources for studying the rose flowering process. Genome 51, 827-837. (IF 1.7, cited 18)
- Foo, E., Bullier, E., Goussot, M., Foucher, F., Rameau, C., and Beveridge, C.A. (2005). The branching gene *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. Plant Cell 17, 464-474. (IF 11.1, cited 92)
- Platten JD, Foo E, Foucher F, Hecht V, Reid JB, Weller JL (2005) The cryptochrome gene family in

pea includes two differentially expressed CRY2 genes. Plant Mol. Biol. 59: 683-696. (IF 3.3, cited 13)

- Hecht, V., Foucher, F., Ferrandiz, C., Macknight, R., Navarro, C., Morin, J., Vardy, M.E., Ellis, N., Beltran, J.P., Rameau, C., and Weller, J.L. (2005). Conservation of Arabidopsis flowering genes in model legumes. Plant Physiol. 137, 1420-1434. (IF 6.1, cited 92)
- Weller, J.L., Foucher, F., Hecht, V., and Rameau, C. (2003). New directions for the genetics of flowering in pea. Flowering Newsletter 36, 15-24.
- Foucher, F., Morin, J., Courtiade, J., Cadioux, S., Ellis, N., Banfield, M.J., and Rameau, C. (2003). DETERMINATE and LATE FLOWERING are two TERMINAL FLOWER1/CENTRORADIALIS homologs that control two distinct phases of flowering initiation and development in pea. Plant Cell 15, 2742-2754. (IF 11.1, cited 71)
- Foucher F. and Kondorosi E. (2000) Cell cycle regulation in the course of nodule organogenesis in *Medicago*, Plant Mol. Biol., 43: 773-786. (IF 3.3, cited 45)
- Charrier B., **Foucher F**., Kondorosi E., d'Aubenton-Carafa Y., Thermes C., Ratet P. (1999) Bigfoot : a new family of MITE elements characterized from the *Medicago* genus, Plant J., 18(4), 431-441. (IF 6.9, cited 25)

1.2 Articles soumis et rapports de recherche

Dubois, A., Carrere, S., Raymond, O., Pouvreau, B., Cottret, L., Roccia, A., Onesto, J.-P., Sakr, S., Atanassova, R., Baudino, S., **Foucher, F.,** Le Bris, M., Gouzy, J. and Bendahmane, M. (soumis) Transcriptome database ressource and gene expression atlas for the rose. *BMC Genomics*.

2. Ouvrages, chapitres d'ouvrages, rapports diplômants

- Foucher, F. (2009). Functional genomics in rose. In Genetics and Genomics of Rosaceae, K.M. Folta and S.E. Gardiner, eds (New york: Springer), pp. 381-392.
- **Foucher F.** (1999) Etude du rôle et de la fonction d'une cycline D3, Medsa;CycD3;1, chez la luzerne Thèse, Institut National Agronomique Paris-Grignon.
- Foucher F. (1995) Etude du devenir des facteurs Nod dans la rhizosphère lors de la symbiose entre Rhizobium meliloti et Medicago sativa, DEA , Université Paris V, XI et INAP-G.

3. Communications dans des congrès et colloques

Orale: (orateur)

- **Foucher F.**, Sakr S., Bendahmane M., Smulder M., Debener T., De Riek J., Torres A. M., Amaya I., Millan T., Zamir D., Sargent D., Nybom H., Atanassov A., Hokanson S., Byrne D., Sosinski B., Main D., Bruneau A., Rees J., Matsumoto S., Yamada K. (2012). The rose genome sequencing initiative Plant & Animal Genome XX, San Diego (CA, USA), 14-18 janvier 2012
- Randoux M., Thouroude T., Juchaux M., Davière J. M., Gaston A., Denoyes B., Iwata H., Hibrand Saint-Oyant, L., Foucher, F. (2012). The continuous flowering regulator, *RoKSN*, is induced by gibberellins to modulate flowering in rose. Plant & Animal Genome XX, San Diego (CA, USA), 14-18 janvier 2012
- Iwata, H., Gaston A., Remay, A., Randoux, M., Thouroude, T., Jeauffre, J., Gala, R., Kawamura, K., Hibrand-Saint Oyant, L., Takashi, A., Denoyes-Rothan, <u>Foucher F.</u> (2010). Blooming and blooming again : what we learn from rose and strawberry. 5th international *Rosaceae* genomics conference. Stellenbosch (South Africa), 14-17 november 2010.

- Gaillard S., Lang T., Franc A., Frigério J.M., Mozar M., Pernet A., Foucher F., Garnier-Géré P., (2009). Automatic treatment of sequences from haploid/diploid DNA, two new tools : SeqQual and ploymorfind. Colloque INRA-EPGV : détection, gestion et analyse du polymorphisme nucléique des génomes végétaux. Evry (France), 11-13 may 2009.
- Djenanne S., Lalanne D., Thouroude T., Sakr S., Leduc N., <u>Foucher F.</u>, Hibrand-saint-Oyant L., (2009) Impact des facteurs environnementaux sur la régulation des gènes MAX/RMS lors du débourrement chez le rosier de jardin. Séminaire COSAVE « Construction et santé du végétal : sélection de résultats marquants des recherches en Région Pays de la Loire ». Angers (France), 10 décembre 2009.
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 - 4. Instruments pour la recherche mis à disposition de communautés scientifiques (logiciels, bases de données, matériel biologique...)

Deux bases de données pour les ESTs ont été développées :

(1) une première suite à la production des 5000 ESTs (Foucher et al, 2008; <u>http://urgi.versailles.inra.fr/GnpSeq/</u>, collaboration avec Fabrice Legeais, URGV, Evry). A partir des 5000 séquences uniques de cette base de données, une puce de type Affymétrix® Rosier a été développée (Collaboration avec Mohammed Bendahmane, ENS Lyon, Financement INRA).

(2) Une seconde base de données suite au projet AIP ((http://iant.toulouse.inra.fr/R.chinensis, Collaboration groupe Nationale Rose et Jérome Gouzy, INRA, Toulouse)

Pour la détection des SNPs à partir de produits PCR d'individus diploïdes hétérozygotes, un pipeline a été développé en collaboration avec Alix Pernet (Diversité génétique du rosier) et Sylvain Gaillard (bioinformaticien, GenHort) :

http://genhort.angers.inra.fr/projects/polymorfind/wiki/index.php/Polymorfind

5. Produits, documents et publications destinés à des utilisateurs de la recherche (professionnels, partenaires institutionnels...)

Brevet :

Rameau, C., Foucher, F., Lejeune-Henaut, I. (2004) « Polypeptides involved in floral development and genes encoding the same », Patent accepted on July, 29th (2004), Publication number WO2004/063378

4. Activités de recherche

Ma carrière scientifique a commencé par les études d'interactions plantesmicroorganismes, qui sont résumées dans la première partie de cette synthèse. Au cours de ces études, j'ai pu appréhender comment les micro-organismes influençaient le développement de la plante, lors des interactions symbiotiques chez la luzerne. Je suis progressivement passé des bactéries aux interactions avec les plantes, puis au développement des plantes. Ainsi, je me suis spécialisé dans le développement des plantes par des approches de génétique classique et fonctionnelle. Ces études ont principalement été réalisées à partir d'approches de type gène-candidat. Il s'agissait, à partir des données disponibles sur les plantes modèles (principalement *Arabidopsis thaliana*), d'étudier la fonction des gènes homologues chez une plante d'intérêt. J'ai ainsi abordé le contrôle du cycle cellulaire chez la luzerne (travaux de thèse), puis le contrôle de la floraison chez le pois (post-doc à l'INRA de Versailles dans le cadre d'un programme Génoplante) et chez le rosier (travaux réalisés à l'INRA d'Angers depuis mon recrutement comme CR2 en 2003). Plus globalement sur le rosier, dans le cadre de l'UMR GenHort (puis maintenant au sein de l'IRHS), je développe un programme sur la génétique et la génomique du rosier.

4.1. Rôle des oligosaccharides dans les interactions plantes-microorganismes

4.1.1. Etude de la transduction du signal lors de l'accumulation des phytoalexines au cours de l'interaction entre le soja et le pathogène *Phytophthora megasperma*

Cette étude a été réalisée dans le cadre d'un stage de 2 mois dans le laboratoire de M.G Hahn (Complex Carbohydrate Research Center, Athens, USA). En réponse à l'agression par le champignon pathogène, *Phytophthora megasperma*, le soja accumule des composés anti-fongiques, les phytoalexines. Cette accumulation a lieu en réponse à un éliciteur produit par le pathogène (Ebel and Grisebach, 1988) qui correspond à une molécule simple, un hepta- β -glucane, issu de la dégradation de la paroi du pathogène par des enzymes végétales (Cheong et al., 1991).

L'objectif de l'étude était de mieux comprendre la transduction du signal en réponse à cet éliciteur et plus particulièrement d'analyser le rôle des pompes H^+ -ATPase par une approche pharmacologique (utilisation d'inhibiteur et de stimulateurs de ces pompes). Cette étude a été réalisée à partir d'un essai biologique sur des cotylédons mis au point dans le laboratoire (Cheong et al., 1991). En utilisant des stimulateurs et inhibiteurs de ces pompes et en variant le pH du milieu extracellulaire, nous avons émis l'hypothèse que l'activation des pompes H^+ -ATPase serait une étape importante dans l'induction de l'accumulation des phytoalexines.

4.1.2. Etude du devenir des facteurs Nod dans la rhizosphère au cours de la symbiose entre *Sinorhizobium meliloti* et *Medicago sativa*

Cette étude a été réalisée dans le cadre de mon stage de DEA de phytopathologie, effectué dans le laboratoire d'Eva Kondorosi (Institut des Sciences Végétales, CNRS, Gif sur Yvette) sous la direction de Michaël Schultze.

Lors de la symbiose entre les Légumineuses et les Rhizobiacées, un dialogue moléculaire se met en place entre la plante et la bactérie, menant au développement de la nodosité fixatrice d'azote. Les facteurs Nod, produits par la bactérie, sont reconnus par la plante et déclenche le programme de nodulation (Foucher and Kondorosi, 2000). Les facteurs Nod sont des molécules lipochito-oligosaccharidiques (figure 1, (Savoure et al., 1994)). Les différences entre les facteurs Nod reposent sur le nombre de résidus *N*-acétyl glucosamines, sur la nature de la chaîne d'acide gras et sur des décorations présentes aux extrémités (comme groupement sulfate ou *O*-acétyl). Ces variations jouent un rôle important dans la spécificité et l'activité des facteurs Nod. Les facteurs en dimères ou trimères inactive les molécules. De plus, une nouvelle activité hydrolytique, induite par les facteurs Nod, formant des dimères lipophiles (dénommée D.F.E., pour Dimer Forming Enzyme) a été mise en évidence (Staehelin et al., 1995).

L'objectif de mon DEA était de comprendre qu'elle pouvait être l'importance de cette dégradation lors de la symbiose. Il s'agissait de suivre le devenir des facteurs Nod dans la rhizosphère (*in planta*) au cours de la nodulation.

Accumulation de NodRmIV(Ac,C16:2,S) au cours de la nodulation

Nous avons déterminé la nature des facteurs Nod produits par la souche utilisée. Elle produit principalement 4 types de facteurs Nod (tableau 1). Le facteur majoritaire est NodRmIV(Ac,C16:2,S) (figure 1). Ces résultats ont été comparés avec ceux obtenus au cours de la nodulation. Il s'agissait de suivre l'évolution des facteurs Nod présents dans la rhizosphère (équilibre entre production et dégradation). Très rapidement (après 48 heures), un seul facteur, NodRmIV(Ac,C16:2,S), est détecté dans la rhizosphère. Ce facteur Nod est le facteur produit majoritairement par la bactérie, mais nous avons aussi montré qu'il s'agissait du facteur le plus stable dans la rhizosphère (tableau 1).

Pour étudier cette stabilité, la dégradation *in vitro* au cours du temps par différentes enzymes (chitinases de classe I et III, DFE) a été testée vis-à-vis des 4 facteurs Nod majoritairement produits (tableau 1). NodRmIV(Ac,C16:2,S) est le facteur le plus stable. Il est dégradé uniquement par la DFE. Cette différence de stabilité entre les facteurs Nod pourrait s'expliquer par les décorations aux extrémités réductrices et non réductrices, telles que la présence du groupe *O*-acétyl.

En conclusion, l'activité à long terme des facteurs Nod, menant à la formation des nodosités, serait déterminée par l'action d'enzymes hydrolytiques. D'autres études ont montré le clivage des facteurs Nod par les enzymes avec des degrés de stabilité différents selon les facteurs Nod (Staehelin et al., 1994; Staehelin et al., 1995; Minic et al., 1998; Schultze et al., 1998; Ovtsyna et al., 2005). Aujourd'hui aucune preuve formelle de l'implication de ces enzymes dans le contrôle de la nodulation n'a été apportée malgré la production de plantes transgéniques surexprimant des chitinases ou des hydrolases (telles que la DFE, communication personnelle de M. Balland). La question reste donc toujours ouverte.

Recherche d'une nouvelle activité modifiant les facteurs Nod.

Suite à la caractérisation des facteurs Nod, un enjeu majeur était l'isolement des récepteurs de ces facteurs et la compréhension de la transduction du signal. Au début de ma thèse, j'ai décidé de m'intéresser à un autre type d'activité enzymatique qui pourrait jouer un rôle dans la perception des signaux Nod. Une activité d'hydrolyse de la chaîne d'acide gras pourrait être impliquée dans la transduction du signal Nod. En effet le micro-bombardement de racines de vesce (*Vicia sativa*) avec des chito-oligosaccharides (sans la chaîne d'acide gras) provoque une induction des divisions cellulaires au niveau du cortex interne, alors que dans le même temps les mêmes molécules appliquées sur la racine sont sans effet (Schlaman et al., 1997). La chaîne d'acide gras des facteurs Nod jouerait un rôle dans la délivrance de la molécule à l'intérieur des cellules (et/ou de la racine). Nous avons émis l'hypothèse qu'une activité acyl-hydrolase pourrait intervenir dans la signalisation des facteurs Nod. L'objectif de ma thèse était ainsi de mettre en évidence une telle activité, et de voir son rôle dans la perception des facteurs Nod.

Après avoir mis en place un test de détection de l'activité par chromatographie gazeuse (détection de la présence de la chaîne d'acide gras), j'ai cherché cette activité enzymatique dans différentes fractions protéiques racinaires. De nombreuses conditions ainsi que différentes acyl-hydrolases purifiées ont été testées, mais aucune activité n'a pu être mise en évidence. Face à cette absence de résultats, j'ai préféré ne pas continuer dans cette voie, l'hypothèse de départ ne pouvant être vérifiée. Les résultats récents montrent que l'hypothèse émise était fausse. En effet, les facteurs Nod seraient perçus par des protéines présentant un motif Lysine associé à un domaine kinase (LysM-RLK). Chez *Medicago truncatula* et *Lotus japonicum*, deux gènes de cette famille (*MtNFP*, *MtLYK3*, *LjNFR5* et *LjNFR1*) sont nécessaires pour l'infection et la nodulation en réponse aux facteurs Nod (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2007). Ces protéines interagissent directement avec les facteurs Nod et seraient ainsi les récepteurs de ces molécules (Broghammer et al., 2012).

4.2. Analyse de l'expression et du rôle d'une cycline D3, MedsaCycD3, chez la luzerne.

J'ai réorienté ma thèse en m'intéressant à la réactivation du cycle cellulaire par les facteurs Nod au cours de la nodulation. Une des thématiques principales du laboratoire d'Eva Kondorosi (ISV, CNRS, Gif/Yvette) était la compréhension de la régulation du cycle cellulaire chez les végétaux en utilisant comme modèle l'organogenèse de la nodosité fixatrice d'azote chez la luzerne. Je me suis intéressé au rôle d'une cycline G1, cycline de type D3 (*Medsa;CycD3*) lors du développement de la plante et lors de la nodogenèse.

4.2.1. Contrôle général du cycle cellulaire

La régulation du cycle cellulaire est un processus complexe. Le cycle cellulaire est divisé en 4 phases. La phase S (Synthèse d'ADN) et M (Mitose) représentent les deux évènements clé, séparés respectivement par les interphases G1 et G2 (G pour Gap) (figure 2). Les points de contrôle sont situés au niveau des transitions G1/S et G2/M. Le passage de ces points se fait par l'intégration de nombreux signaux endogènes (taille de la cellule) et

exogènes (présence de facteurs de croissance). Ces transitions sont régulées par l'activation de kinases spécifiques : les CDK (Cyclin Dependent Kinases, dénommées également CDC2), qui interagissent avec des sous-unités régulatrices, les cyclines. L'activité du complexe CDK-cycline est finement régulée par l'interaction avec des protéines inhibitrices, les CKI (CDK inhibitors) et par différentes phosphorylations et/ou déphosphorylations. De façon générale (mais avec quelques exceptions), les CDK sont exprimées constitutivement au cours du cycle cellulaire. L'expression des cyclines est régulée avec différentes vagues (figure 2a) : cyclines mitotiques (dites A et B) et les cyclines G1 (dites D et E).

Au début de ma thèse (1995), les mécanismes de contrôle du cycle cellulaire commençaient juste à être élucidés chez les végétaux. Le clonage et l'étude de nombreux gènes impliqués dans le contrôle du cycle cellulaire ont permis de montrer que sa régulation était assez semblable à celle observée chez les animaux (pour revue voir (Stals et al., 2000; Stals and Inze, 2001)). Ainsi de multiples CDK (Joubès et al., 2000), ainsi que des cyclines mitotiques, type A et B (Chaubet-Gigot, 2000) et des cyclines G1, type D (Meijer and Murray, 2000) ont été clonées chez les végétaux. La figure 2a présente les différents gènes impliqués dans le contrôle du cycle cellulaire chez la luzerne. Les CDK sont activés par l'interaction avec des cyclines, dont l'expression est régulée au cours du cycle cellulaire. D'autres éléments régulateurs ont pu être mis en évidence tels que la phosphorylation et déphosphorylation des CDK (Zhang et al., 1996; Sun et al., 1999), la dégradation des cyclines (Genschik et al., 1996) ou du facteur de transcription E2F (Ramirez-Parra et al., 1999).

Ainsi de façon assez similaire aux animaux, la transition G2/M serait contrôlée par la formation d'un complexe MPF (Maturation Promotion Factor) comprenant un CDK et une cycline A ou B. Ce complexe serait activé par phosphorylation et déphosphorylation du CDK. En fin de mitose, le complexe serait désactivé par dégradation des cyclines mitotiques via l'APC (Anaphase Promoting Complexe). De même il a été proposé que les cyclines D interagiraient avec une CDK en phase G1 et permettraient la phosphorylation de la protéine Rb et le relargage du facteur de transcription E2F, nécessaire à la transcription des gènes de la phase S (figure 2b).

Ces études ont permis de comprendre les mécanismes moléculaires du contrôle du cycle cellulaire sur les végétaux. Il est également important de comprendre comment le cycle cellulaire est intégré dans le développement de la plante (interaction avec les hormones telles qu'auxines ou cytokinines). Quelques éléments de réponse commencent à être apportés avec par exemple les cytokinines et les cyclines D (figure 2b). Comme chez les animaux, les cyclines D joueraient un rôle d'intégration des signaux et seraient le lien entre hormone et réactivation du cycle cellulaire. Une cycline D3 chez *Arabidopsis* est activée par les cytokinines, et sa surexpression peut se substituer aux cytokinines (Riou-Khamlichi et al., 1999).

Le développement de la nodosité fixatrice d'azote est un modèle pour étudier l'intégration du cycle cellulaire dans les programmes de développement végétal (Foucher and Kondorosi, 2000). En effet, il est possible de suivre la dédifférenciation des cellules du cortex en réponse au signal Nod, et la mise en place d'un nouveau méristème d'où les cellules quittent le cycle cellulaire pour entrer en différenciation. Il s'agit de comprendre comment en réponse aux facteurs Nod la réactivation du cycle cellulaire mène à la formation du méristème nodulaire et comment les cellules en prolifération quittent le cycle cellulaire pour se différencier en cellules fixatrices d'azote.

Pour répondre à ces questions nous avons choisi de travailler sur la plante modèle *Medicago truncatula* et je me suis intéressé à l'implication possible d'une cycline D3, MedsaCycD3 (Dahl et al., 1995) dans l'organogenèse de la nodosité. Des études antérieures montraient que les cellules du cortex interne étaient bloquées au stade G0/G1 et ré-entraient

dans le cycle cellulaire par une synthèse d'ADN (Savoure et al., 1994; Yang et al., 1994). De par leur rôle lors de la transition G1/S, les cyclines D3 pourraient être le lien entre les facteurs Nod et la réactivation du cycle cellulaire.

4.2.2. Objectifs et approches développées lors de la thèse

Les objectifs de mon travail de thèse étaient d'analyser le rôle de la cycline D3 lors de l'organogenèse de la nodosité et d'étudier l'implication de cette cycline dans le développement de la plante.

Deux approches ont été développées pour répondre à ces questions :

(1) Analyse de l'expression de la cycline par suivi de l'activité GUS dans des plantes contenant le gène rapporteur *uidA* fusionné aux promoteurs de la cycline, par hybridation *in situ*, par Northern et par RT-PCR

(2) Analyse fonctionnelle par surexpression de la cycline chez *Medicago truncatula*.

4.2.3. Caractérisation des séquences génomiques de la cycline D3 chez *Medicago sativa* et *Medicago truncatula*.

L'analyse par Southern génomique (hybridation avec la sonde ADN) a montré qu'il existe au moins 3 copies du gène *MedsaCycD3* chez *Medicago sativa* (Ms) et une seule copie chez *Medicago truncatula* (Mt). Après criblage d'une banque d'ADN génomique, nous avons isolé 2 clones indépendants chez Ms et un clone chez Mt. Les deux cyclines isolées chez *M. sativa* ont été dénommées MsI et MsII et celle chez *M. truncatula* Mt (figure 3).

Les principales différences entre les 3 séquences se situent dans la zone 5' (zone dite promotrice). Entre MsI et MsII, on note la présence de 4 insertions chez MsII (figure 3). L'une de ces insertions (*Msbf2*) est un élément transposable du type MITE (Minuscule Inverted Transposable Element) et a fait l'objet d'une publication (description des éléments MITE, *bigfoot* chez *Medicago* (Charrier et al., 1999)). Une des autres insertions présentait des homologies avec une séquence non codante de tabac. La séquence Mt est assez semblable sur les 500 premières paires de bases (pb) en amont de +1 de transcription avec la séquence MtI. On note la présence de l'insert 2 et d'un nouvel insert propre à Mt (insert Mt). La suite de la séquence est divergente, on ne retrouve plus d'homologie avec les séquences de MsI ou II (figure 3).

Les régions 5' promotrice de MsII et I ont été fusionnées de façon traductionnelle avec le gène rapporteur GUS et les constructions ont été introduites chez *Medicago truncatula* par tranformation via *Agrobacterium tumefaciens*.

4.2.4. Analyse de l'activité GUS des plantes transgéniques promoteur CycD3-GUS

Pour chaque construction, plus de 12 plantes indépendantes (validées par Southern génomique) ont été régénérées. Une première analyse rapide de l'expression GUS à la génération T0 en culture *in vitro* a montré que les 2 promoteurs (MsI et II) présentaient des profils d'expression différents. Il semble que MsI s'exprime dans les tissus en division (cals ou pointes racinaires) et MsII dans les tissus en différenciation (feuille, racine). Pour préciser ces résultats, l'expression GUS a été étudiée à la génération suivante (génération T1).

Nous avons analysé l'expression GUS au cours du développement de la plante et au cours de la nodogenèse. Les résultats obtenus ont été vérifiés par d'autres techniques (Northern, hybridation *in situ* ou RT-PCR). En effet, les résultats obtenus avec le GUS peuvent être parfois artéfactuels.

La première difficulté à laquelle nous avons été confrontés était l'hétérogénéité des profils observés pour une même construction. Il était assez difficile de définir un profil majoritairement représentatif. Sans entrer dans les détails, avec les plantes MsII, on observe trois types de profil (pas d'expression, expression attendue, profil aberrant) représentant un tiers des plantes respectivement. Pour la suite de l'étude, nous n'avons conservé que les plantes présentant un profil « attendu » (profil obtenu par d'autres approches comme l'hybridation *in situ*), mais on ne doit pas oublier que ces plantes représentent seulement un tiers des plantes régénérées.

Le second problème concerne l'expression dans les racines. Aucun signal GUS n'est détecté avec le promoteur MsII, et seulement un signal au niveau de la coiffe avec le promoteur MsI, signal qui disparaît rapidement au cours du développement de la racine. Ce résultat est surprenant car la cycline D3 avait été décrite comme s'exprimant dans l'apex racinaire (Dahl et al., 1995). Nous avons vérifié par Northern et par hybridation *in situ* le profil d'expression de la cycline au niveau de la racine. On détecte un faible signal par Northern et par hybridation *in situ* au niveau de l'apex racinaire (figure 4a et b). De plus par Northern sur les racines des plantes transgéniques, aucun transcrit du gène *uidA* (GUS) n'est détecté (figure 4a), confirmant l'absence d'activité GUS dans les racines. Cette absence montre un comportement anormal des plantes. Des éléments régulateurs importants pour l'expression de la cycline dans les racines seraient absents. Ces éléments pourraient être présents dans les introns ou la partie 3'UTR du gène.

Dans les parties aériennes de la plante, on détecte une activité GUS semblable pour les deux constructions : dans l'apex caulinaire (zone méristématique), dans les jeunes feuilles (l'activité n'est plus détectable dans les feuilles matures) et dans les fleurs (étamines et carpelles). Au cours de la germination, la cycline D3 est exprimée dans la graine après imbibition (embryon et cotylédon). Cette expression diminue avec la croissance de la plante et se limite aux cotylédons. Ces données montrent une régulation de l'expression de la cycline au cours du développement de la plante.

Au cours du développement de la nodosité, les promoteurs présentent deux profils distincts. Aucun signal GUS n'est détecté avec le promoteur MsI, alors qu'un signal est détecté avec les plantes MsII (Figure 5a). Ce signal est localisé dans la nodosité mature dans les zones I (méristème), zone II et interzone II-III (zone en différenciation). Ces résultats ont été confirmés par hybridation *in situ* (figure 5b). L'expression de la cycline D3 dans des zones en différenciation laisse supposer un nouveau rôle pour cette cycline, qui ne serait pas uniquement lié à la prolifération cellulaire. Ces résultats ouvrent de nouveaux horizons pour les cyclines D3 dans des processus de différenciation. Elles pourraient être impliquées dans les phénomènes d'endoréduplication qui ont lieu lors de la différenciation des cellules de la nodosité.

Aucun signal n'a pu être détecté dans les premiers stades de développement de la nodosité par Northern, hybridation *in situ* ou dans les plantes promoteur-GUS (figure 5). Pour détecter un faible signal, nous avons analysé l'expression de la cycline D3 par RT-PCR et montré une induction précoce de la cycline (après 9h) et de façon transitoire (jusqu'à 4 jours) avec des amorces spécifiques de la cyclineD3-MsI. Il y a donc une activation transitoire de la cycline D3 dans les étapes précoces de la nodogenèse telles que la réactivation du cycle cellulaire (les premières divisions sont visibles après 48h). Une cycline D3 (MsI) interviendrait donc précocement lors de la réactivation du cycle cellulaire (hypothèse de départ), puis une autre (MsII) plus tardivement lors de la différenciation (figure 6, (Foucher and Kondorosi, 2000).

Nous avons étudié la différence d'expression entre les promoteurs MsI et MsII dans la nodosité, et recherché l'élément responsable de l'expression dans la nodosité. Pour cela, des délétions successives du promoteur MsI ont été réalisées. Les résultats obtenus, ainsi que les

comparaisons de séquences entre MsI et II, ont permis de supposer que l'élément responsable serait l'insert 3 (figure 3). Pour tester cette hypothèse, des expériences de gel retard ont été réalisées entre l'insert 3 et des extraits protéiques de nodosités. Différentes conditions ont été testées, mais aucun retard sur gel n'a pu être mis en évidence.

4.2.5. Etude des plantes surexprimant la cycline D3 en orientation sens.

La cycline D3 a été introduite chez *M. truncatula* sous le contrôle d'un promoteur fort (35S) en orientation sens. Une quinzaine de plantes indépendantes a été régénérée et étudiée. La surexpression du gène a été vérifiée dans les feuilles par Northern. Différents niveaux d'expression ont été obtenus, le plus souvent supérieur à la plante sauvage.

Une analyse phénotypique détaillée des plantes a été réalisée. Aucun problème n'a été rencontré lors de la régénération. Le développement des plantes était semblable aux plantes sauvages. Toutes les plantes formaient des nodosités fixatrices. Pour vérifier un rôle possible de la cycline dans les processus d'endoréplication, nous avons regardé le niveau de ploïdie dans les nodosités, aucune différence n'a été détectée. De plus aucun effet sur la croissance racinaire en présence de phytohormones (auxine ou cytokinines) n'a été mis en évidence. La surexpression de la cycline D3 ne provoque pas de phénotype majeur, ceci pourrait s'expliquer par une régulation post-transcriptionnelle (dégradation rapide de la protéine, interaction avec le CDK, phosphorylation).

Ces résultats sont différents de ceux obtenus chez *Arabidopsis*, où la surexpression de la cycline D3 provoque différents phénotypes (problème de régénération, feuilles anormales, absence de besoin de cytokinines pour l'embryogenèse somatique (Riou-Khamlichi et al., 1999)). Il semble donc que la cycline D3 d'*Arabidopsis* et celle de *Medicago* aient des fonctions ou régulations différentes.

4.2.6. Conclusions

Lors de cette étude, deux cyclines D3 ont été caractérisées chez *M. sativa* présentant des profils d'expression différents lors de la nodulation : MsI s'exprimerait lors de la réactivation du cycle cellulaire et MsII lors de la différenciation (figure 6). Ces données laissent supposer un nouveau rôle de la cycline lors des processus de différenciation (nodulation ou développement de l'embryon). Ce nouveau rôle n'a pu être vérifié par l'approche fonctionnelle (absence de phénotype majeur chez les plantes surexprimant la cycline). Nous avons également montré que la cycline D3 était impliquée dans le développement de différents organes.

Il pourrait donc exister deux types de cycline D3 chez les végétaux avec des fonctions différentes : l'une impliquée dans la réactivation du cycle cellulaire et la perception de signaux (facteurs Nod ou cytokinines) et l'autre impliquée dans la différenciation. Chez *Arabidopsis*, les cyclines CYCD3 (CYCD3-1; CYCD3-2 et CYCD3-3) jouent un rôle dans l'équilibre entre cycle mitotique et d'endoreduplication (Dewitte et al., 2007). CYCD3 restreindrait l'endoreduplication et intégrerait les signaux hormonaux (auxine et cytokinine).

Suite à ma thèse où j'ai acquis des compétences en génétique fonctionnelle ainsi qu'une connaissance du développement et des Fabacées, j'ai été recruté pour travailler sur la floraison d'une autre Fabacée le pois par des approches de génétique.

4.3. Contrôle de la floraison chez les plantes : importance des gènes de la famille TFL1 chez le pois, une plante monocarpique, et chez le rosier, une plante polycarpique.

4.3.1. Le contrôle de la floraison chez les plantes : un modèle de référence : *Arabidopsis thaliana*.

La floraison est un processus complexe mais essentiel pour la survie et la multiplication des plantes. Son contrôle est ainsi finement régulé par des facteurs environnementaux (photopériode, froid, quantité de lumière, température ambiante) et des facteurs endogènes (comme les hormones, principalement les gibbérellines, ou le développement de la plante). La floraison correspond au passage d'un méristème végétatif à un méristème floral, menant à la formation d'une fleur (ou d'une inflorescence). Ce processus peut être décomposé en plusieurs étapes (Figure 7):

* <u>l'induction florale</u> correspond à l'acquisition de la compétence à fleurir en réponse aux facteurs environnementaux et/ou endogènes.

* <u>l'initiation florale</u> est marquée par les changements morphologiques observés au niveau du méristème.

* <u>le développement floral</u> correspond au développement des différentes pièces florales (sépales, pétales, étamines et carpelles).

Ces différentes étapes font intervenir de nombreux gènes interagissant entre eux et avec les différents facteurs environnementaux et endogènes. A partir de mutants, les études approfondies menées chez *A. thaliana* ont permis de mettre en évidence un réseau génique complexe, qui aujourd'hui sert de bases pour l'étude des bases moléculaires de la floraison chez de nombreuses plantes. Ces nombreuses études ont été largement synthétisées (Amasino, 2010; Fornara et al., 2010; Turnbull, 2011; Andres and Coupland, 2012). Pour une compréhension des résultats présentés, je vais brièvement décrire les principaux résultats et surtout m'attarder sur la famille des gènes *TERMINAL FLOWER 1 (TFL1)/ FLOWERING LOCUS T (FT)*.

4.3.1.1. Un réseau génique complexe contrôlé par de multiples voies

A. thaliana est une plante de jours longs (LD) facultative. Chez certains écotypes (Winter annual), une période de froid, vernalisation, accélère la floraison. La température ambiante accélère également la floraison. *Arabidopsis* fleurit plus vite à 23°C qu'à 16°C (Lee et al., 2007). Les gibbérellines (GA) stimulent la floraison avec un effet plus important en jours courts (SD), conditions photopériodiques non inductibles.

Au cours de l'induction florale (IF), les gènes d'initiation florale sont induits et activent les gènes d'identité florale (*LEAFY (LFY), APETALA1 (AP1)* et *FRUITFUL (FUL)*)) qui marquent la conversion du méristème végétatif en méristème floral (initiation florale, figures 7 et 8). Les gènes d'identité florale en retour vont mener à l'induction des gènes d'identité des organes floraux (modèle ABCE, (Irish, 2010)). Deux gènes de l'IF jouent un rôle clé, il s'agit des intégrateurs floraux : *FT* et *SUPRESSOR OF CONSTANS 1 (SOC1)*. Ces deux gènes intègrent différentes voies environnementales (photopériode, vernalisation, température ambiante) et endogènes (âge de la plante, voie autonome, gibbérellines). L'ensemble de cette régulation complexe est partiellement décrite en détail dans la figure 8.

En réponse à la photopériode, le facteur de transcription *CONSTANS* est accumulé en LD dans les feuilles et active la transcription de *FT* et de son paralogue *TSF (TWIN SISTER OF FT)*. *FT* (et *TSF*) sont des activateurs de la floraison (Kardailsky et al., 1999; Kobayashi et al., 1999; Yamaguchi et al., 2005) et sont une des composantes du florigène¹ (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Notaguchi et al., 2008). Ainsi les protéines FT et TSF migrent des feuilles via le phloème vers l'apex où elles interagissent avec un facteur de transcription FD (FLOWERING LOCUS D, (Abe et al., 2005; Wigge et al., 2005). Le complexe FD/FT induit les gènes *SOC1, AP1* et *FUL*. L'induction de *AP1* pourrait être indirecte via *SOC1* qui induiraient les facteurs de transcription de la famille *SPL (SQUAMOSA PROMOTER BINDING PROTEIN LIKE*, (Jung et al., 2012)), qui induisent les gènes d'identité florale (Yamaguchi et al., 2009).

FT est la cible de nombreuses régulations. Il est ainsi induit par la photopériode via CO. Il est réprimé par le facteur de transcription de type boîte MADS, FLC (FLOWERING LOCUS C). FLC est un répresseur central de la floraison chez *Arabidopsis* (pour revue, (Kim et al., 2009)). Ce répresseur est réprimé par la vernalisation et la voie autonome. Chez les écotypes d'*Arabidopsis* qui ont un besoin de vernalisation, en absence de vernalisation, *FLC* est fortement exprimé et accumulé. Cette expression nécessite la présence d'un allèle actif au locus *FRIGIDA* (*FRI*). En réponse au froid, le niveau de transcription de *FLC* diminue. Des modifications de la chromatine mènent à l'absence de transcription de *FLC*. *FLC* est également réprimé par la voie autonome. *FLC* réprime la transcription de *FT* en formant un complexe avec un autre facteur de transcription de type boîte MADS, SVP (SHORT VEGETATIVE PHASE, (Li et al., 2008)). *SVP* intervient également dans le contrôle la floraison par la température ambiante (Lee et al., 2007).

Les gibbérellines (GA), et principalement GA₄, activent la floraison chez *Arabidopsis* majoritairement en SD (Eriksson et al., 2006). GA₄ interviendrait à plusieurs niveaux dans le contrôle de la floraison. Dans les feuilles, GA₄ induirait l'expression de *CO* en LD (Hisamatsu and King, 2008; Porri et al., 2012). Dans l'apex, les GAs auraient comme cible *SOC1* et *LFY* par une voie indépendante de CO/FT. L'activation de *LFY* ferait intervenir un facteur de transcription de type MYB33 régulé par les GA (Gocal et al., 2001; Eriksson et al., 2006). En réponse au GA, la protéine DELLA est dégradée et permet la libération du facteur de transcription (SPL) qui permettrait l'induction de *LFY* (Yu et al., 2012). L'activation de *SOC1* par les GA passerait par une répression de *SVP* (Moon et al., 2003; Li et al., 2008). Avant l'initiation florale, GA₄ s'accumule dans les feuilles et migre vers l'apex. Cette accumulation ne corrèle pas avec une modification du métabolisme des GA (Eriksson et al., 2006).

L'âge de la plante fait intervenir une régulation de 2 miRNA (*miR156* et *miR172*). *miR156* promeut la phase juvénile en réprimant les gènes de la famille *SPL* (Wu and Poethig, 2006; Gandikota et al., 2007), qui induisent *miRNA172* (Wu et al., 2009). La surexpression de *miR156* retarde la floraison et étend la phase juvénile (Wu and Poethig, 2006; Wu et al., 2009). Progressivement au cours du développement de la plante, *miRNA156* est réprimé via un signal provenant des feuilles (Yang et al., 2011) et *miRNA172* est accumulé (passage de la phase juvénile à la phase adulte). En absence de *miRNA156*, les facteurs de transcription SPL s'accumulent et l'accumulation de *miRNA172* réprime des répresseurs floraux de la famille AP2 tels que *SMZ* qui répriment directement ou indirectement *FT* (Mathieu et al., 2009).

Ce réseau génique de contrôle de la floraison fait ainsi intervenir à la fois des activateurs et des répresseurs floraux qui répondent aux différentes voies endogènes et

¹ La notion de florigène a été développée à partir des travaux de Knott (1934) qui a montré que la photopériode était perçue au niveau des feuilles. Cette étude posait la question de la migration d'un signal des feuilles vers l'apex pour induire la floraison. Des études ultérieures ont montrés que ce stimulus floral passait les points de greffe et était universel. Il fut dénommé florigène par Chailakhyan (1936)

environnementales. Au final, le signal est intégré au niveau de l'apex. Au niveau de l'apex, une autre protéine TFL1 intervient dans le devenir du méristème.

4.3.1.2. Importances des gènes de la famille TFL1 / FT dans le développement des plantes.

TFL1 (TERMINAL FLOWER 1) est une protéine de la même famille que FT. Cette famille de gènes a été dénommée CETS pour *CENTRORADIALIS/TERMINAL FLOWER1/SELF PRUNING*. Chez *Arabidopsis*, cette famille comprend 5 gènes *FT*, *TSF* (paralogue de *FT*), *TFL1*, *ATC (ARABIDOPSIS THALIANA CENTRORADIALIS)*, *BFT (BROTHER OF FT)* et *MFT (MOTHER OF FT AND TFL1)*. Comme nous venons de le voir, *FT et TSF*, 2 paralogues, sont des activateurs de la floraison et joue un rôle central comme intégrateurs floraux et composante du florigène. FT et TSF sont des régulateurs du facteur de transcription FD en formant un complexe protéique (Abe et al., 2005). Chez le riz, ce complexe ferait intervenir une protéine de type 14.3.3 qui permettrait la localisation nucléaire de la protéine FT (Taoka et al., 2011). De même, *MFT* est un activateur floral. Sa surexpression provoque une floraison précoce, toutefois le mutant *mft* ne présente pas de phénotype (Yoo et al., 2004).

Dans la famille CETS, des gènes ont une fonction antagoniste et répriment la floraison. Le plus étudié de ces gènes est TFL1. Le mutant tfl1 présente une floraison précoce et une croissance déterminée de l'inflorescence (mise en place rapidement d'une fleur terminale, figure 9a). Ainsi TFL1 contrôlerait l'identité du méristème en réprimant la transition florale et en maintenant l'identité du méristème inflorescenciel (Bradley et al., 1997; Ratcliffe et al., 1998). TFL1 est principalement exprimé dans le méristème médulaire caulinaire avec une augmentation de son expression lors de l'initiation florale (Bradley et al., 1997; Ratcliffe et al., 1998). La protéine est mobile et se retrouve dans l'ensemble du méristème caulinaire (Conti and Bradley, 2007). La présence de TFL1 maintiendrait ainsi la croissance indéterminée de l'inflorescence, sauf sur les flancs du méristème où se développent les primordia floraux et où les gènes d'identité floral (comme LEAFY et APETALA1) sont exprimés (figure 9b). Une régulation complexe existe entre TFL1 et LFY/AP1. La surexpression d'AP1 provoque la répression de TFL1 (Liljegren et al., 1999) alors que le triple mutant ap1 cal ful présente une forte expression de TFL1 (Ferrandiz et al., 2000). AP1 régulerait directement l'expression de TFL1 en se fixant sur des éléments cis (boîte CArG) situés en aval du gène (Kaufmann et al., 2010). La mutation de ces sites provoque un phénotype semblable aux mutants tfl1. De même un mutant dominant négatif de LFY provoque une expression ectopique de TFL1 dans les primordia floraux, expression corrélant avec une réversion de l'inflorescence (Parcy et al., 2002). L'expression de TFL1 dans le méristème apical permet de maintenir une croissance indéterminée de celui-ci, alors que sur les flancs du méristème, la régulation négative de TFL1 par AP1 et LFY permet la formation d'un méristème floral à croissance déterminée.

La régulation de *TFL1* par les facteurs endogènes ou environnementaux est peu connue. *TFL1* interviendrait dans la réponse à la température ambiante. En effet le mutant *tfl1* présente une faible différence de floraison entre 16 et 23°C. TFL1 jouerait un rôle répressif principalement via *SOC1* (Strasser et al., 2009).

Concernant le mode d'action de TFL1, TFL1 agit comme un répresseur transcriptionnel et cette répression nécessite la présence de la protéine FD (Hanano and Goto, 2011). Une hypothèse serait une compétition entre FT et TFL1 pour l'interaction avec FD (Abe et al., 2005; Wigge et al., 2005). La formation d'un complexe FD/FT activerait la floraison, alors que la formation d'un complexe FD/TFL1 aurait une activité répressive. Cette différence d'activité reposerait sur un seul acide aminé essentiel. Ainsi la substitution de cet

acide aminé chez TFL1 (H88Y) et son acide aminé correspondant chez FT (Y85H) modifie l'activité répressive de TFL1 en activateur et inversement chez FT (Hanzawa et al., 2005). La substitution de cet acide aminé affecte la structure des protéines, modification d'une loupe externe (Ahn et al., 2006). Au niveau cellulaire, TFL1 est localisé majoritairement dans le cytoplasme (Conti and Bradley, 2007), toutefois un signal est détecté également dans le noyau en système hétérologue (Hanano and Goto, 2011). En présence de FD, la protéine est relocalisée dans le noyau (Hanano and Goto, 2011). On peut supposer que comme pour Hd3a (l'homologue de FT chez le riz), TFL1 interagit avec une protéine 14.3.3 qui relocalise la protéine dans le noyau et permet l'interaction avec FD (Taoka et al., 2011). De plus, TFL1 est présent dans les compartiments endo-membranaires et interviendrait dans le trafic des vacuoles du stockage protéique (Sohn et al., 2007). Le mutant *tfl1* présente un défaut au niveau de ce trafic.

Deux autres gènes de la famille CETS ont une activité répressive comme TFL1. Il s'agit d'ATC et de BFT. ATC est un répresseur floral ; sa surexpression permet la complémentation du mutant tfl1. Toutefois, le mutant atc ne présente pas de phénotype. ATC est uniquement exprimé dans l'hypocotyle de jeunes plantules (Mimida et al., 2001). Les plantes surexprimant BFT ont une floraison retardée et présentent des problèmes de développements floraux assez semblables à ceux observés lors de l'expression ectopique de TFL1. Le mutant bft ne présente pas de phénotype alors que le double mutant tfl1 bft présente un phénotype au niveau de l'inflorescence, suggérant une fonction redondante entre TFL1 et BFT pour le développement de l'inflorescence (Yoo et al., 2010).

Au cours d'une étude chez le pois et le rosier, j'ai abordé la fonction des gènes de la famille *TFL1* chez ces deux espèces. A partir d'une approche de type 'gène-candidat', l'objectif était d'étudier la fonction des gènes de cette famille et leur évolution chez une espèce monocarpique et polycarpique.

4.3.2. DETERMINATE et LATE FLOWERING, deux gènes impliqués dans l'architecture de l'inflorescence et le contrôle de la floraison, sont des paralogues du gène TFL1.

Lors d'un post-doctorat réalisé dans le cadre d'un projet Génoplante Pois, s'intitulant "Développement d'une carte de référence de gènes de fonction connue chez le pois". Dans le groupe de Catherine Rameau (INRA, Versailles), j'ai participé au développement de marqueurs de gènes de fonction connue impliqués dans la précocité de floraison (principale activité) et dans l'architecture du pois (encadrement d'Erika Bullier dans le cadre de son stage de DEA). Un des objectifs majeurs de l'INRA en ce qui concerne la culture de pois est le développement de pois d'hiver. Cette amélioration passe par une meilleure résistance au froid mais aussi par un évitement des gelées de printemps, en jouant sur les dates d'initiation florale. En effet tant qu'une plante n'a pas initié la floraison, cette plante est plus résistante au froid. Ce processus est connu sous le nom d'échappement au froid ("freezing escape"). Toutefois, la floraison ne doit pas être trop tardive pour éviter le développement et le remplissage de la graine lors des fortes chaleurs d'été. Une régulation fine, et donc une connaissance du contrôle de la floraison, sont nécessaires. La physiologie de la floraison du pois est bien documentée et l'utilisation de nombreux mutants avait permis de proposer des modèles de contrôle de la floraison impliquant des répresseurs et des activateurs floraux (figure 10a, (Reid et al., 1996; Weller et al., 1997)). La synthèse d'un stimulus floral est sous le contrôle de GIGAS (Beveridge and Murfet, 1996). La synthèse d'un inhibiteur floral est régulée par la photopériode et sous le contrôle de différents gènes (STERILE NODE, HIGH RESPONSE, PHOTOPERIOD and DAY NEUTRAL). Au niveau de l'apex, le gène LATE *FLOWERING (LF)* intègre les différents signaux et détermine le nœud de floraison chez le pois (Figure 10a et b). Quatre classes d'allèles, *Lf-d, Lf, lf et lf-a*, ont été définies pour le gène *LF* conférant respectivement dans les conditions inductives un nœud minimal de floraison de 15, 11, 8 et 5 (Murfet, 1975). Après l'initiation florale, le pois développe une inflorescence indéterminée (Figure 10b). Le mutant *determinate (det)* produit rapidement une fleur terminale apparente (Singer et al., 1990). En fait, une étude fine par microscopie a permis de montrer que la fleur terminale était en fait axillaire et que le méristème cessait son activité et entrait en sénescence (Singer et al., 1990).

J'ai confronté les modèles physiologiques développés chez le pois avec les modèles moléculaires développés pour l'initiation florale chez *Arabidopsis* (voir introduction). La même approche a été développée par l'équipe de J. Weller (Hobard, Australia) sur les gènes du développement floral. L'ensemble a permis d'avoir une vision globale de la conservation des gènes de floraison entre *Arabidopsis* et le pois (Hecht et al., 2005). Ainsi la plupart des gènes sont conservés chez le pois et *Arabidopsis*, même si certains gènes semblent avoir évolué différemment (comme *TERMINAL FLOWER 1, TFL1*). De plus, plusieurs gènes importants, comme *FRIGIDA* et *FLOWERING LOCUS C* sont absents (Hecht et al., 2005). Une forte synténie a été montrée entre le pois et la légumineuse modèle *Medicago truncatula*, montrant des transferts possibles entre plantes d'intérêt agronomique et plantes modèles.

Parmi les gènes isolés, une caractérisation plus précise a été réalisée sur les cryptochromes, photorécepteur de la lumière bleu. Il existe 3 cryptochromes chez le pois (*CRY1, CRY2a* et *CRY2b*). Les protéines codées par les gènes *CRY2* comportent une partie C terminal plus courte et les 3 gènes sont différentiellement régulés (Platten et al., 2005).

Les résultats les plus intéressants ont été obtenus en étudiant des homologues du gène TFL1. Nous avons isolé 3 homologues de TFL1 chez le pois, PsTFL1a, PsTFL1b et PsTFL1c. Par cartographie et séquençage de mutants, nous avons démontré que PsTFL1a et PsTFL1c correspondait au gène DETERMINATE (DET) et LATE FLOWERING (LF) respectivement (Foucher et al., 2003). Le séquençage de 3 allèles det a permis de mettre en évidence 3 mutations responsables d'une perte de fonction. DET contrôle l'architecture de l'inflorescence et son expression est limitée au méristème apical après l'initiation florale (Singer et al., 1990). LF retarde la floraison en allongeant la phase végétative. La variation allélique à ce locus est un élément important de la variation naturelle de la floraison chez le pois (Murfet, 1975). Nous avons montré que les allèles les plus sévères (lf-a, floraison très précoce) portent une délétion ou une mutation menant à la synthèse d'une protéine non fonctionnelle. Les allèles intermédiaires ne présentent pas de modification de la protéine, la différence s'explique par une régulation transcriptionnelle différente du gène : lors de la transition florale, une faible quantité de transcrit de LF corrèle avec une floraison précoce, et une forte quantité de transcrit de LF corrèle avec une floraison tardive (Foucher et al., 2003). Les plantes portant les allèles de floraison précoce (lf) accumulent moins de transcrits LF que les plantes portant les allèles de floraison tardive (LF-d, LF). Ainsi chez le pois, deux homologues de TFL1, DET et LF contrôlent deux phases distinctes de développement alors qu'un même gène, TFL1, contrôle les deux phases chez Arabidopsis thaliana (Figure 10c). Cette étude montre comment deux organismes ont pu développer différentes stratégies pour contrôler leur développement.

Ces études sur la famille TFL1 ont été récemment complétées par une étude des homologues de FT par l'équipe de Jim Weller (University of Tasmania, Australia). Les gènes FT chez les Fabacées sont représentés par trois clades. Un homologue de FT (PsFTa1) correspond au gène GIGAS. Le mutant gigas présente une absence de floraison en LD et un retard en SD. GIGAS contrôle la production d'un signal induisant la floraison et transmissible par greffe (Figure 10a, (Beveridge and Murfet, 1996; Hecht et al., 2011)). Une épistasie complète de LF sur GIGAS a été mise en évidence. Les mutants gigas lf et lf ont exactement le même phénotype. GIGAS stimulerait la floraison via une régulation négative de LF (Hecht

et al., 2011). Cette régulation négative pourrait passer par une compétition de *LF* (homologue de *TFL1*) et de *GIGAS* (homologue de *FT*) vis-à-vis de la protéine FD, comme proposé chez *Arabidopsis* (Abe et al., 2005; Wigge et al., 2005)

Au cours de ce post-doctorat, j'ai également participé à l'encadrement d'une étudiante de M2 sur le contrôle de la ramification en caractérisant l'expression d'un gène clé *RAMOSUS1 (RMS1)*, un orthologue de *MORE AXILLARY GROWTH 4 (MAX4)*. Nous avons montré un gradient d'expression du gène dans les bourgeons axillaires le long de la tige (Foo et al., 2005). Il a été montré récemment que ce gène intervenait dans la synthèse d'une nouvelle hormone, les strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008).

4.3.3. Contrôle de la floraison chez le rosier.

Suite à ce post-doc, j'ai été recruté en tant que CR2 à l'INRA d'Angers pour développer un programme sur l'architecture du rosier. L'étude réalisée sur le pois m'a permis de voir l'importance de combiner une approche génétique et moléculaire pour caractériser des gènes-clé du développement. Ce type d'approche a été développé sur le rosier **pour comprendre les bases moléculaires et génétiques de l'architecture aérienne du rosier buisson, et plus particulièrement le contrôle de la remontée de floraison.** Le contrôle de la floraison est un enjeu majeur en horticulture ornementale, où la valeur d'une plante repose principalement sur sa fleur. Je développerai dans un premier temps les résultats obtenus sur l'importance du gène *TFL1* dans le contrôle de la remontée de floraison, avant de développer plus largement les approches de génétique et génomique développées sur le rosier.

Concernant la floraison, les principales études ont été menées chez les plantes monocarpiques (Arabidopsis thaliana, riz, pois...), les études sont encore peu nombreuses chez les plantes polycarpiques, plantes pouvant avoir plusieurs floraison successive. Le rosier est un modèle original pour l'étude de la floraison chez les plantes polycarpiques. En effet la plupart des plantes polycarpiques ont une floraison par an, or chez le rosier, certains génotypes peuvent refleurir plusieurs fois par an. Ce processus est appelé remontée de floraison. Cette remontée peut avoir lieu occasionnellement (souvent à l'automne) ou tout le long de l'année (floraison continue, figure 11a). La floraison continue est contrôlée par un gène majeur récessif (RB pour RECURRENT BLOOMING, (Semeniuk, 1971)) introduit à partir des rosiers de Chine (Ogisu, 1996). Des études physiologiques antérieures avaient montré que l'apport exogène de gibbérellines (GA) au printemps inhibaient la floraison des rosiers non remontants, mais étaient sans effet sur les rosiers remontants (Roberts et al., 1999). De plus, nous avons montré que chez le rosier non remontant, l'induction florale a lieu au printemps très rapidement après la reprise de croissance, alors que chez de nombreuses Rosacées (Fraisier, Pommier, Pêcher) l'induction florale a lieu à l'été avant l'entrée en dormance du bourgeon. Au niveau moléculaire, chez le rosier, l'induction florale est marquée par l'induction d'un homologue de FT, puis de LFY (Remay et al., 2009). Au niveau morphologique, on observe la conversion du méristème végétatif en un méristème floral (Foucher et al., 2008). Dans le cadre d'une approche gène candidat, nous avons émis deux hypothèses pour le contrôle de la remontée de floraison chez le rosier : implication de répresseurs floraux (qui bloqueraient de nouvelles floraisons) et implication de la voie des GA.

4.3.3.1. KSN, un homologue de TFL1 contrôle la remontée de floraison chez le rosier et la fraise des bois.

Nous avons montré que le gène contrôlant la floraison continue est codé par un répresseur floral, dénommé RoKSN, appartenant à la famille de TFL1 (Iwata et al., 2012). Chez les rosiers à floraison continue, RoKSN présente une insertion d'un rétrotransposon (de type copia) dans le second intron du gène (Figure 11b). La présence de ce rétrotransposon provoque un épissage alternatif et aucun transcrit fonctionnel du gène RoKSN n'a pu être détecté chez les rosiers à floraison continue. Les rosiers à floraison continue produisent régulièrement des mutants à refloraison occasionnelle (mutation de type 'Climbing', (Lewis, 1994)). Cette mutation (en fait une réversion) est expliquée par la recombinaison du rétrotransposon et la restauration d'un allèle fonctionnel chez six couples de mutants d'origines variées (Figure 11b). L'ensemble de ces résultats nous a permis de conclure que RoKSN, un homologue du gène TFL1 contrôlait la floraison continue chez le rosier (Iwata et al., 2012). L'expression de RoKSN est régulée au cours du développement avec une expression faible lors de la première floraison et une forte expression dans les tiges végétatives. Ainsi, une forte expression du gène est liée à une absence de floraison alors qu'une faible expression est associée avec l'initiation florale. De plus, nous avons étudié la remontée de floraison chez la fraise des bois (F. vesca), une espèce proche du rosier où une remontée de floraison existe également. Chez la fraise des bois, la floraison continue est contrôlée par un gène majeur récessif, SEASONNAL FLOWERING (SLF). Nous avons montré que les loci SFL et RB sont synténiques entre le fraisier et le rosier. De plus, chez F. vesca, une délétion de deux paires de bases dans la région codante de l'homologue de RoKSN, FvKSN, est proposée comme responsable de la remontée de floraison. Ces résultats ont été obtenus en collaboration avec Ikaru Iwata (Wakeningen Co; Hiroshima, Japan) et Béatrice Denoyés-Rothan (UREF, INRA, Bordeaux). Une équipe finlandaise a confirmé les résultats obtenus chez le fraisier par une validation fonctionnelle chez Arabidopsis et le fraisier (Koskela et al., 2012). L'extinction du gène FvKSN par RNAi permet la remontée de floraison chez un fraisier non remontant. Les auteurs proposent une régulation négative de FvKSN par les jours courts (SD), expliquant la floraison en SD du fraisier des bois (Koskela et al., 2012). Ces résultats sont controversés car d'autres auteurs décrivent les fraisiers des bois à floraison continue comme ne répondant plus à la photopériode (Battey et al., 1998) ou comme des plantes de jours longs (Sonsteby and Heide, 2008).

La validation fonctionnelle du gène *RoKSN* est en cours. Nous avons complémenté le mutant *tfl1* d'*Arabidopsis* (floraison tardive et croissance indéterminée) par surexpression de *RoKSN* (Marie Randoux, non publié). Des rosiers transgéniques surexprimant *RoKSN* ont été obtenus en collaboration avec les entreprises Delbard. La transformation a été réalisée sur un rosier remontant. Trois plantes indépendantes ont été obtenues (validée par Southern blot). Elles présentent une accumulation importante de transcrits de *RoKSN* (qRT-PCR) et de la protéine RoKSN (Western blot). Après plusieurs mois en serre S2, ces plantes n'ont pas fleuri alors que les témoins (vecteur vide) présentent rapidement une inflorescence. Ainsi la surexpression de *RoKSN* chez un rosier remontant bloque la floraison. *RoKSN* est bien un inhibiteur de la floraison (Marie Randoux, résultats non publiés).

4.3.3.2. KSN est sous le contrôle transcriptionnel des GA.

L'autre hypothèse concernant le contrôle de la remontée de floraison implique les GA. Nous avons isolé chez le rosier les gènes impliqués dans la signalisation et le métabolisme des GA. Deux homologues de gènes importants de la signalisation, *RoDELLA* et *RoSPINDLY*, se localisent génétiquement proches du gène de la remontée de floraison et d'un QTL contrôlant la date de floraison (Remay et al., 2009). L'analyse précise des séquences n'a pas pu permettre de valider leur implication dans le contrôle de la floraison. Pour valider la fonction de *RoDELLA*, nous avons surexprimé le gène chez le pélargonium, plante ornementale facilement transformable et présentant une remontée de floraison (Hamama et al., in press). La surexpression de *RoDELLA* bloque ou retarde la floraison. D'autres effets sur le développement des pélargoniums ont été observés : réduction de la taille, ramification et formation des racines (Hamama et al., in press).

Nous avons isolé des homologues de gènes impliqués dans le métabolisme de GA : 2 homologues de gènes clé de la synthèse des formes actives des GA ; *RoGA200X* et *ROGA30X* et un gène impliqué dans l'inactivation des GA, *RoGA20X* (Remay et al., 2009). Nous avons proposé que le métabolisme des GA soit impliqué dans le contrôle de la floraison chez les rosiers. Ainsi une dégradation des GA serait nécessaire avant l'initiation florale (induction d'un gène codant une enzyme de dégradation des GA, et répression d'un gène impliqué dans la synthèse, Figure 12a,(Remay et al., 2009)). Ces données corrèlent avec des données antérieures concernant l'évolution du contenu en GA au cours du processus de floraison : faible contenu lors de l'induction florale et augmentation de la concentration en GA lors de l'initiation et le développement floral (Roberts et al., 1999)

Robert et al. (1999) avaient également montré que les rosiers à floraison continue étaient insensibles aux GA. L'apport exogène de GA n'inhibait pas la floraison. Nous avons étudié plus en détail le lien entre GA et *RoKSN*. Chez les rosiers non remontants, les GA inhibent la floraison au cours de l'induction florale, période où l'intégrateur floral *RoFT* est normalement induit. En présence de GA, on observe une forte accumulation des transcrits de *RoKSN*, une réduction de l'accumulation des transcrits de *RoFT* et *RoSOC1* et une absence d'accumulation des transcrits *RoLFY*, *RoAP1* et *RoAP1* (Randoux et al., in press). Cette régulation transcriptionnelle de *RoKSN* par les GA a été validée dans d'autres systèmes (culture de nœuds) et l'analyse du promoteur du gène (par expression transitoire dans des feuilles de tabac) a permis de mettre en évidence la présence d'élément *cis* contrôlant la réponse aux GA (Randoux et al., in press). L'inhibtion florale par les GA serait ainsi médié via l'induction de *RoKSN*. Chez les rosiers remontants (en absence de GA3, uniquement *RoLFY* et *RoFD* sont réprimés, suggérant une régulation indépendante de *RoKSN* pour ces deux gènes.

Les résultats actuels nous permettent de proposer un modèle de contrôle de la floraison du rosier intégrant le gène de la remontée de floraison et les GA (Figure 12b et 12c). Ainsi au printemps, une faible teneur en GA pourrait permettre de maintenir un niveau faible de transcrits de l'inhibiteur floral *RoKSN* et ainsi la floraison pourrait avoir lieu. En présence de GA, le gène *RoKSN* s'accumule et interagirait avec FD et bloquerait l'accumulation des transcrAprès cette floraison, le niveau de *RoKSN* est élevé, et de nouvelles floraisons sont impossibles. Cette accumulation des transcrits de *RoKSN* pourrait être liée à la présence de GA. Toutefois, l'apport exogène d'un inhibiteur de synthèse des GA, le paclobutrazol, ne permet pas une floraison, mais réduit la taille des entrenoeuds (Randoux et al., in press). D'autres facteurs (tels que le froid) pourrait intervenir dans le contrôle de *RoKSN*. Chez les rosiers à floraison continue, du fait de l'absence de *RoKSN*, quelles que soient les conditions (présence ou absence de GA) et la période, l'inhibiteur est absent et la floraison a lieu. La répression de *RoLFY*, indépendante de *RoKSN*, n'est pas suffisante pour réprimer la floraison. Ces résultats montrent un rôle important du gène de la remontée de floraison aussi bien lors de la remontée de floraison que lors de la floraison de printemps.

4.3.3.3. Existence d'autres voies de contrôle de la floraison chez le rosier.

Pour compléter l'approche gène-candidat (qui repose sur des hypothèses), nous avons également développé une approche sans *a priori* basée sur la comparaison du transcriptome au cours de l'induction florale chez deux rosiers non remontants, *R. x wichurana* et *R. hybrida* 'Félicité et Perpétue'. Pour cela, nous avons utilisé la puce Affymétrix® Rosier développée dans le cadre d'une collaboration avec l'ENS de Lyon (voir le chapitre 'Développement de ressources génétiques et génomiques').

Pour étudier précisément l'induction florale et l'initiation florale, nous avons caractérisé les modifications morphologiques au cours de ces processus. Après le débourrement, le méristème est étroit et en forme de dôme avec présence de primordia foliaires sur ses flancs, une structure typique d'un méristème végétatif (stade VM1 pour méristème végétatif). Rapidement, le méristème s'élargit et les primordia foliaires ne sont plus visibles (Stade VM2, pour 'pré-floral'). Ensuite, le méristème devient floral, avec élargissement et aplatissement, le premier verticille (sépale) pouvant être visible (stade FM pour 'méristème floral' (Dubois et al., 2011). Nous avons comparé le transcriptome de ces 3 stades chez les deux rosiers non remontants en nous intéressant plus particulièrement à la transition entre VM1 et VM2 (induction florale).

Chez *R. wichurana* (W) et 'Félicité et Pérpétue' (FP), 824 et 652 gènes respectivement sont différentiellement exprimés (DE) entre les stades VM1 et VM2. Pour augmenter la confiance dans la découverte des gènes, nous avons analysé les gènes DE en commun entre W et FP. Nous en avons ainsi mis en évidence 222 gènes présentant un profil semblable entre FP et W dont 131 gènes sont induits entre VM1 et VM2 et 91 sont réprimés (Dubois et al., 2011). Parmi les gènes induits, on retrouve *RoAP1* et *RoSOC1* dont on avait déjà montré qu'ils étaient induits au cours de la floraison (Remay et al., 2009). Cette approche n'a pas permis de mettre en évidence de gène impliqué dans la voie des GA (principale hypothèse précédemment étudiée). Toutefois, six gènes impliqués dans le transport et la signalisation de l'auxine sont dérégulés au cours de l'induction florale (Dubois et al., 2011). Ces résultats suggèrent une signalisation active de l'auxine au cours de l'induction florale. Chez *Arabidopsis thaliana*, un rôle de l'auxine a été montré uniquement au cours du développement de l'inflorescence ; le méristème inflorescentiel du mutant *pin1* ne fait pas de fleur (Reinhardt et al., 2000). De plus, 2 gènes impliqués dans la signalisation de l'éthylène sont réprimés, suggérant un rôle synergique entre l'éthylène et l'auxine.

Récemment, nous avons développé de nouvelles EST et réalisé du RNASeq (expression *in silico* des gènes) chez un rosier remontant, *R. chinensis* 'Old Blush' à partir de différents tissus, dont des tissus au cours de l'initiation florale (voir le chapitre suivant). Comme lors de l'approche transcriptomique, *RoSOC1* et *RoAP1* sont induits au cours de la floraison. De même, trois gènes de signalisation de l'auxine, deux gènes de signalisation de l'éthylène et deux gènes du métabolisme de l'éthylène sont dérégulés au cours de l'induction florale. Comme proposé chez des rosiers non remontant (Dubois et al., 2011), l'auxine et l'éthylène pourrait jouer un rôle dans le contrôle de la floraison chez un rosier remontant (Dubois et al., soumis).

L'ensemble de ces résultats suggèrent un rôle possible de l'auxine et de l'éthylène au cours de l'induction florale. Des expériences complémentaires sont nécessaires pour valider cette hypothèse.

4.4. Génétique et Génomique de l'architecture du rosier.

4.4.1. Développement de ressources génétiques et génomiques.

Les outils de génétique ont été principalement développés par Laurence Hibrand-Saint Oyant (IR dans l'équipe). Je ne développe que brièvement cet aspect. De nouveaux marqueurs co-dominants (microsatellites) ont permis d'étoffer la carte génétique (Hibrand-Saint Oyant et al., 2008). Ces études ont été complétées par la réalisation d'une carte consensus rosier (Spiller et al., 2011) au niveau international en collaboration avec T. Debener (University of Hannover, Germany), D. Byrne (Texas AM University, USA) et R. Schmulder (PRI, The Netherland). En parallèle, des études de synténie ont été réalisées entre le fraisier (*F. vesca*) et le rosier, deux plantes très proches (Section des Rosoïdées dans la famille des Rosacées). Nous avons montré une colinéarité bien conservée entre ces deux plantes et plus particulièrement au niveau du locus contrôlant la remontée de floraison (Iwata et al., 2012).

En 2003, au commencement du projet Rosier à Angers, peu (ou pas) d'outils de génomique existait chez le rosier. Afin de développer des études de transcriptomiques, nous avons produit des EST chez un rosier remontant (R. hybrida 'Black Baccara') et non remontant (R. x wichurana) à partir de tiges au cours de l'initiation florale (Foucher et al., 2008). Les EST ainsi produites ont été assemblées avec les EST disponibles dans les bases de données (Channeliere et al., 2002; Guterman et al., 2002) et ont permis d'obtenir un premier jeu de 5000 séquences uniques qui ont servi de bases à la construction d'un puce Affymétrix[®], utilisée pour étudier l'initiation et le développement floral en collaboration avec M. Bendahmane (INRA, RDP, Lyon) (Dubois et al., 2011). Ces données ont été complétées en 2009 par la production d'un nouveau jeu d'EST dans le cadre d'un projet national (AIP Bioressources), regroupant l'ensemble des équipes publiques françaises travaillant sur le rosier: RDP (Lyon), GenHort (Angers), SAGAH (Angers), IMEP (Marseille), URIH (Antibes), BV-PAM (St Etienne), CNRGV (Toulouse), LIPM (Toulouse). Ce séquençage d'EST a été réalisé à partir de banques normalisées obtenues à partir de différents tissus de R. chinensis 'Old Blush' (séquençage 454). Nous disposons ainsi aujourd'hui de 67 000 contigs. Ce séquençage 454 a été complété par une approche RNASeq via un séquençage SOLEXA de chacune des banques non normalisées. L'ensemble des résultats ainsi produit est disponible en ligne (dB Rosa: http://iant.toulouse.inra.fr/plants/rosa/portal/cgi/rosa.cgi). Pour chaque contig, une annotation précise a été réalisée et une expression virtuelle (comptage des séquences SOLEXA) est disponible (voir discussion sur les résultats floraison transcriptomique). Ces résultats sont en cours de publication (Dubois et al., soumis).

En vue d'une validation fonctionnelle, un travail important a été engagé pour développer des protocoles rapides et efficaces de transformation génétique du rosier. En effet le rosier est une plante récalcitrante à la transgenèse (Debener and Hibrand-Saint Oyant, 2009). En collaboration avec Philippe Vergne (INRA, ENS Lyon), les développements sont en cours sur des rosiers remontants et non remontants dans le cadre du projet Genius (investissement d'avenir) avec l'implication de Latifa Hamama (IR, Université d'Angers) et Laurence Hibrand-Saint Oyant.

4.4.2. Bases génétiques de l'architecture du rosier

Ce projet est développé en collaboration avec les écophysiologistes de l'UMR SAGAH. Il fait partie du projet structurant du centre INRA d'Angers-Nantes et a été soutenu par la région « Pays de la Loire » dans le cadre du programme « COSAVE », dont j'étais l'animateur de l'axe 2 « Fonctionnement des structures précoces au cours du développement des plantes »

Au niveau de la plante entière, nous avons réalisé une étude génétique sur les composantes architecturales (Post-doctorat de 2 ans de Koji Kawamura, financé par l'INRA). Une méthodologie adaptée au matériel végétal a été développée en collaboration avec Evelyne Costes (INRA, Montpellier) pour phénotyper la forme de la plante, la ramification et l'architecture de l'inflorescence. Cette étude a été réalisée sur une nouvelle descendance (R. x wichurana X 'The Fairy') pour laquelle une carte génétique a été développée. Ainsi plus de 60 caractères ont été analysés. Ces caractères complexes sont très héritables et contrôlés majoritairement par 6 zones du génome, dénommées cQTL, pour QTL commun (Kawamura et al., 2011). Des co-localisations intéressantes ont été mises en évidence entre ces QTL (Figure 13). Ainsi le locus contrôlant la remontée de floraison (RoKSN / RB) joue un rôle majeur dans l'architecture de la plante. L'homologue de FT (RoFT) co-localise avec un QTL intervenant dans la date de floraison (cette co-localisation est confirmée sur une autre descendance (Hanae Roman, M2, 2012)). Un autre QTL co-localise avec l'homologue de RoFD. Ainsi les gènes de la famille TFL1 (ou des partenaires de ces gènes) pourraient jouer un rôle important dans le développement des plantes, rôle non limité au contrôle de la floraison. Enfin, un QTL contrôlant le degré de ramification de l'axe primaire co-localise avec un homologue de MAX2, gène impliqué dans la signalisation des strigolactones, hormones contrôlant la ramification (Figure 13).

Au niveau des processus, nous nous sommes intéressés au gradient de ramification (Post-doctorat de 1 an de Samia Djennane dans le cadre du projet COSAVE). Après un traitement à la lumière et au froid, nous avons mis en évidence une modification de ce gradient chez le rosier. Cette étude a été complétée par une approche moléculaire avec l'isolement des gènes homologues des gènes *MAX* (*MORE AXILLARY MERISTEM*) impliqués dans le contrôle de la ramification chez *Arabidopsis*. Dans le traitement lumière, le gradient de débourrement corrèle avec l'expression des gènes *MAX2* (perception des strigolactones). Ces résultats sont en cours de valorisation (publication en cours de rédaction).

5. Description de l'expérience dans l'encadrement et l'animation d'une recherche

5.1. Encadrement des étudiants, doctorants et post-doctorants.

Le tableau 2 présente la liste des étudiants que j'ai encadrés au cours de ma carrière. Le devenir de ces étudiants est également présenté. La liste des mémoires des travaux que j'ai encadrés est présentée dans le chapitre 5.6 (Liste des travaux encadrés).

J'ai encadré un étudiant en thèse : Arnaud Remay (2005-2009) sur « les bases moléculaires de la floraison chez le rosier » (Soutenue le 17 septembre 2009, Université d'Angers). Le directeur de thèse était Mathilde Briard (Professeur à Agrocampus Ouest), j'ai réalisé l'encadrement scientifique et technique au quotidien. De plus j'encadre scientifiquement depuis fin 2009 une nouvelle thèse (Marie Randoux, « Contrôle moléculaire de la remontée de floraison chez le rosier », Agrocampus-Ouest). Cette thèse est dirigée par Noëlle Dorion (Professeur émérite à Agrocampus Ouest). La thèse d'Arnaud Remay a mené à la publication de trois articles dans des revues à comité de lecture, dont deux en premier auteur. La thèse de Marie Randoux a mené à la publication d'un article en première auteur.

J'ai encadré 2 post-doctorants : Koji Kawamura (« Génétique quantitative de l'architecture du rosier de jardin : analyse architecturale et étude du déterminisme génétique », 2007-2009, financement INRA) et Samia Djenanne (« Impact des facteurs environnementaux sur la régulation des gènes *MAX/RMS* lors du débourrement chez le rosier de jardin », 2009, projet COSAVE, financement régional). Samia Djenanne a été recrutée sur un projet conjoint avec l'UMR SAGAH (Angers), et a été co-encadrée avec Soulaiman Sakr et Nathalie Leduc (UMR SAGAH). Dans le cadre du post-doc de Koji Kawamura, deux articles ont été accepté, dont un en premier auteur. Concernant Samia Djenanne, un article est en cours de rédaction. Lors de mon post-doc à Versailles, j'ai encadré une Ingénieur d'étude sous contrat (Julie Morin).

Au cours de ma carrière, j'ai eu l'occasion d'encadrer des étudiants en M2 (11 étudiants) et L3 (2 étudiants). Ces encadrements ont pu être conjoints avec des membres de l'équipe : Laurence Hibrand-Saint Oyant (Ingénieur de recherche), Alix Pernet (Ingénieur de recherche), Vanessa Soufflet-Freslon (Maître de conférences) et Sylvain Gaillard (Bioinformaticien). Je participe à l'élaboration et la programmation du sujet de recherche, au suivi des expérimentations, à l'analyse des résultats, à la correction du rapport et à la préparation de l'exposé. Les étudiants sont associés aux publications de l'équipe si la contribution est significative.

5.2. <u>Animation scientifique</u>

Au sein de l'unité mixte de recherche :

Au sein de l'UMR GenHort (Génétique et Horticulture), depuis mon recrutement en 2003, j'ai assuré l'animation d'un petit groupe « Architecture du Rosier », regroupant une ingénieur de recherche (Laurence Hibrand-Saint Oyant), un technicien en génétique fonctionnelle (David Lalanne, remplacé en 2009 par Julien Jeauffre, suite à une mobilité), une technicienne de terrain (Tatiana Thouroude, recrutement en 2005, 50%), un technicien de culture *in vitro* (Gilles Michel, 50%) et une technicienne en marquage génétique (Annie Chastellier, recrutement en 2010, 50%). L'animation du groupe se fait en étroite relation avec l'ingénieur. L'ingénieur est plus responsable des approches de génétique quantitative et je gère plus les approches de génétique fonctionnelle. Progressivement, nous avons essayé

d'élargir ce groupe en nouant des relations avec d'autres personnes de l'UMR pour développer des approches de validation fonctionnelle chez le rosier (Noëlle Dorion (Agrocampus Ouest), Latifa Amama (Université d'Angers)), et des études sur la diversité génétique du rosier (Alix Pernet, INRA ; Florence Daguin, Université d'Angers). Ceci a mené à la mise en place d'une animation scientifique hebdomadaire commune sur les activités de recherche concernant le rosier au sein de l'UMR (discussion des résultats, des projets, présentation d'articles...).

Ce groupe appartient à l'équipe « Ornement » qui était animée jusqu'en 2009 par Véronique Kapusta. Depuis 2009, j'ai pris l'animation de l'équipe « Ornement » (une vingtaine de personnes) qui regroupe les activités de recherche sur le rosier (« Architecture du rosier », « Diversité génétique ») et des activités sur le genêt (Phylogénie et développement d'outils d'aide à la sélection). Dans ce cadre, j'ai coordonné pour le groupe la rédaction du bilan de l'équipe et le projet de la future équipe au sein de l'IRHS pour l'évaluation par l'AERES.

Depuis 2012, au sein de l'IRHS (UMR « Institut de Recherche en Horticulture et Semences »), je co-anime avec Agnès Grapin (Enseignant-Chercheur à Agrocampus Ouest) l'équipe GDO (Genetics and Diversity of Ornamentals). Cette équipe, qui comprend 25/30 personnes, regroupe l'ensemble des activités menées à l'IRHS sur la génétique des plantes ornementales. Les enjeux de l'équipe sont de comprendre, de gérer et d'utiliser la diversité génétique. Le projet de l'équipe se décline sous forme de 3 questions :

(1) Quels sont les bases génétiques et moléculaires des principaux caractères ornementaux. Nous nous intéressons à l'architecture (et plus particulièrement à la floraison) et à la résistance aux maladies foliaires (projet Rosa fortissima). Mon activité de recherche sur l'étude de la floraison s'insère dans cette thématique.

(2) Quels sont les impacts des pratiques humaines et de la sélection naturelle sur la diversité des espèces du genre *Rosa*? Une partie des travaux concernant cette question est abordée dans le cadre d'une étude sur la remontée de floraison (projet SIFLOR) et dans le cadre d'un projet pluridisciplinaire sur les facteurs de succès de l'innovation variétale chez le rosier aux XVIII^e et XIX^e siècles en France (projet FLORHIGE). Ces projets sont décrits en détail dans la partie « Prospective ».

(3) Comment utiliser les données de diversité génétique en sélection ? Cette thématique est principalement développée dans le cadre du projet BRIO (Breeding Research and Innovation on Ornamentals).

Au niveau national :

Plusieurs équipes sont impliquées dans des travaux sur le rosier. Ces équipes sont réunies au sein du Groupe National Rose qui se réunit tous les 2 ans pour échanger et mettre en place des projets communs. L'équipe GDO est un élément moteur de ce groupe. Nous avons organisé en 2007 la réunion de ce groupe à Orléans. En 2008, un projet AIP Bioressource (INRA) a été porté par le groupe et a permis le séquençage d'EST chez le rosier.

Pour réfléchir sur les enjeux de la filière ornementale, en lien avec les collègues de l'UMR SAGAH (nouvelle équipe ArchE de l'IRHS), nous avons organisé une réunion de la filière ornement (avec des représentants des obtenteurs, des producteurs, des distributeurs, des économistes et les chercheurs de l'IRHS). Cette réunion s'est tenu le 25 janvier 2011.

En 2010, j'ai été nommé par le chef de département « Génétique et Amélioration des Plantes », animateur du groupe Ornement du DGAP (INRA). Je suis membre du groupe filière « Ornement » de l'INRA.

Au niveau international :

Je me suis impliqué activement dans la mise en place d'un groupe international pour promouvoir la génomique et la génétique du rosier. Notre équipe a ainsi organisé en 2007 le premier meeting international sur la génomique du rosier (1st International Rose Genomics Meeting, Angers, 11-13 décembre 2007, 80 participants). Au cours de cette réunion a été décidée la création d'un consortium international sur la génomique du rosier dans lequel je suis membre du « steering committee ». L'un des objectifs principaux de ce groupe est de promouvoir le séquençage du génome de rosier. Par rapport à cette initiative les équipes françaises sont leaders (GDO et ArchE à Angers ; RDP à Lyon).

Animation de projet

Dans le cadre d'un projet régional COSAVE (Construction et Santé du Végétal, 2005-2009), j'ai été l'animateur de l'axe 2 « Fonctionnement des structures précoces au cours du développement des plantes ». Depuis 2012, je suis responsable du projet SIFLOR, « Signature de sélection et déterminisme génétique de la remontée de floraison chez le rosier » (30k€, financé par le département de génétique et d'amélioration des plantes de l'INRA). A partir de 2013, je serai responsable du projet FLORHIGE, « Facteurs de succès de l'innovation variétale du rosier aux XVIII^e et XIX^e siècles en France : démarche interdisciplinaire génétique et historique » (270k€, financé par la région Pays de la Loire dans le cadre des appels d'offre 'Paris scientifiques').

Cette HDR est aussi l'occasion de faire le bilan sur les projets déposés depuis mon recrutement à l'INRA (Annexe 1). La liste est longue, et interroge sur la course et la compétition que mènent les chercheurs aujourd'hui pour décrocher des fonds. Une analyse retrospective montre un taux d'échec assez important les premieres années et un taux de réussite plus important depuis 2 années. Ces réussites s'expliquent aujourd'hui principalement par la reconnaissance du groupe en génétique et génomique du rosier et sur une meilleure qualité des publications. Les principaux soutiens financiers que nous avons eus sur le rosier sont ceux de l'INRA (principalement BV/GAP), la région Pays de la Loire et sur des travaux en collaboration avec les professionnels (CTPS, CASDAR). Les tentatives auprès de l'ANR ont tout été veine et pose la question sur le financement de travaux finalisés sur l'ornement par cette instance.

5.3. Réflexion sur l'encadrement de la recherche

L'encadrement des jeunes chercheurs devrait être aujourd'hui une priorité de tous. Nous devons collectivement faire un effort pour attirer les jeunes vers la recherche (et ce à tous les niveaux du technicien au chercheur). Une partie du problème repose sur des choix politiques. Il s'agit de l'effort national qui doit être fait pour consolider les parcours professionnels en recherche. La précarité des carrières en recherche n'est pas un facteur incitant les jeunes à s'engager dans cette voie. Une programmation pluriannuelles de l'emploi scientifique serait un signal fort envoyé aux jeunes générations et pourrait les inciter à s'engager en recherche. Combien d'étudiants hésitent à s'engager dans une thèse car l'avenir est trop incertain.

Promouvoir la culture scientifique

L'attrait vers les carrières scientifiques peut commencer très jeune. La démarche scientifique est une démarche rigoureuse qui doit être un des piliers de la culture scientifique. Dans un monde incertain, la démarche scientifique, la raison sont des phares pour nous éclairer le monde. Les initiatives qui favorisent la culture scientifique sont à encourager. Les
personnels de la recherche doivent s'engager activement dans la promotion de la culture scientifique. Les initiatives telles que celles développées par « Terre des Sciences » ou les « Petits débrouillards » sont à multiplier. La participation à la promotion de la culture scientifique devrait être prise en compte dans l'évaluation des chercheurs et faire clairement partie de leur mission. Il n'y pas d'âge pour se cultiver. Je voudrais citer ces quelques exemples de ce que peut être cette démarche au niveau personnel:

* Accueil de classes de maternelle. Ce fut l'occasion d'aborder avec des mots simples le travail d'un scientifique, le développement d'une plante et de commencer à faire germer de futures vocations...

* Participation au congrès des jeunes chercheurs par les écoles privées du Maine et Loire. Cette manifestation permet à des élèves de cycle III (CE2, CM1, CM2) de se rencontrer autour d'une problématique expérimentale (le défi). Chaque classe travail pour résoudre la question et lors d'une assemblée plénière les enfants confrontent leurs résultats. C'est l'occasion pour des chercheurs de discuter avec les jeunes, leurs questions très libres sont l'occasion de discuter les métiers et la démarche scientifiques.

* Travaux de vulgarisation (rédaction d'articles vers un large public, comme par exemple 'Têtes Chercheuses') et participation à des opérations de communication (Fête de la science).

Former les jeunes aux métiers de la recherche.

La culture scientifique est la première pierre de l'édifice. Elle doit permettre aux jeunes d'avoir envie de s'orienter vers une carrière scientifique. Notre rôle de chercheurs et d'enseignants chercheurs est d'accompagner ces jeunes. L'accompagnement passe bien sûr par l'accueil dans nos laboratoires sous forme de stage en les formant aux pratiques de laboratoire et à la démarche scientifique. Il est évident qu'un stagiaire n'est pas une main d'œuvre à tout faire, mais hélas il est souvent bon de le rappeler. Le mouvement des stagiaires a permis de mettre en évidence les dérives (et la recherche y participait) et de créer un cadre juridique concernant les stages. Il est toutefois de notre responsabilité de maître de stage que de proposer les conditions optimum de formation et de travail des stagiaires. Accepter un jeune en stage est un contrat moral entre le jeune et l'encadrant. Le jeune s'engage dans le travail qui lui est proposé. L'encadrant s'engage à être à ses cotés, à le former, à lui transmettre ses connaissances. Je conçois la recherche souvent comme une forme d'artisanat, nous sommes les artisans de la connaissance. La recherche est une démarche, des expériences, mais c'est aussi la curiosité, l'envie de comprendre, d'expliquer. Encadrer un jeune chercheur, c'est aussi lui donner cette envie, ce regard critique qui ne doit pas se limiter à la surface, mais toujours creuser pour comprendre. Le tableau 2 présente le devenir des étudiants que j'ai encadrés au cours de ma carrière.

Former de jeunes chercheurs : le pari du doctorat.

Les doctorants représentent un cas à part dans la formation des jeunes scientifiques. Il s'agit d'une formation sur la durée qui doit amener progressivement l'étudiant à devenir un chercheur. Il s'agit d'un vrai pari : pari pour le doctorant qui a décidé de se lancer dans une carrière scientifique longue et souvent incertaine, mais aussi pari pour l'encadrant qui propose un sujet de thèse (au début proposé par l'encadrant, le sujet doit progressivement devenir celui du doctorant).

Un des challenges de la thèse est de progressivement s'effacer par rapport au doctorant, ou bien au doctorant de progressivement s'imposer par rapport à son encadrant, pour que le doctorant puisse exprimer sa créativité et devenir maître d'œuvre de son projet. L'autonomie du doctorant passe par une acquisition progressive de son sujet de thèse avec un accompagnement (synthèse bibliographie), par la gestion des expérimentations (mise

en place, réalisation, collecte des données et leur analyse). Ce dernier point est souvent plus difficile lorsque l'on travaille sur des plantes pérennes, car les expérimentations sont souvent planifiées avant le début de la thèse. Ainsi dans le cadre de la thèse d'Arnaud Remay, les plans d'expérimentation concernant l'analyse transcriptomique étaient déjà bouclés au moment où Arnaud a commencé sa thèse. Toutefois l'analyse des résultats de transcriptomique (technique d'analyse, choix des gènes, étude de ces gènes) a permis à Arnaud d'effectuer des choix et de tester des hypothèses.

Dans le cadre de cette formation qu'est la thèse, le doctorant doit participer / se former à l'ensemble des futures missions d'un chercheur. Il y a bien sûr le dessin, la réalisation et l'analyse des expériences avec un regard critique. La participation à des congrès est également un moment important pour les doctorants. Dans les deux thèses encadrées, les doctorants ont eu l'occasion de présenter oralement en anglais leurs travaux de thèse lors de congrès internationaux. La rédaction d'articles scientifiques est aussi une étape essentielle et nécessaire. J'ai toujours laissé les doctorants être maître d'œuvre dans la rédaction de l'article en les orientant, les recadrant. La réflexion sur le sujet de thèse, sur le travail du chercheur, sur sa place dans la société est aussi un élément important de la formation. Je regrette l'arrêt par l'INRA de la formation « Réflexive » qui permettait pendant une semaine en binôme avec le doctorant ce recul et cette réflexion salutaire sur notre travail. Enfin le travail d'encadrement ne s'arrête pas la thèse finie. Il y a le suivi des valorisations (qui peuvent parfois prendre plusieurs années) et du devenir du doctorant.

Enfin le doctorant doit s'intégrer au sein d'une équipe, intéragir avec les différents membres de l'équipe (techniciens, chercheurs). Ainsi les doctorants participent en lien avec la technicienne qui en a la charge à la mise en place et au suivi du matériel végétal. Le doctorant peut également être amené à encadrer des étudiants. Lors des deux thèses, que j'ai encadrées, le doctorant a co-encadré avec moi des étudiants de M2. Le projet proposé aux étudiants de M2 s'inscrivait dans le cadre de la thèse.

5.4. Mission d'expertise

Je réalise des « reviews » (3 par an en moyenne) pour des articles soumis dans différentes revues, comme Plant Cell, Plant Science, Planta, Plant Breeding, Theoretical and Applied Genetics, Plant Cell Tissue and Organ Culture. J'effectue régulièrement (1 par an en moyenne) des évaluations pour des appels d'offre pour des régions (Aquitaine), des universités (Lyon), des organismes (BARD)...

Participation à des comités de recrutement, comité pilotage, jury thèse J'ai été membre (comme examinateur) de la thèse de

* Marion Maene « Morphogénèse florale chez le rosier : identification et caractérisation de gènes impliqués dans le phénomène de la fleur double et optimisation d'un protocole de transformation génétique stable », Ecole Normale Supérieur de Lyon, soutenue en juin 2008

* Ronan Fernandez « Etude du déterminisme du sexe et du développement floral chez le melon », Ecole Doctorale « Science du Végétal », Ecole doctorale « Science du Végétal », Université Paris-Sud XI, soutenue le 14 décembre 2010

* Amélia Gaston « Etude et compréhension du déterminisme génétique et moléculaire de la remontée florale chez le fraisier », Ecole doctorale des sciences de la vie et de la santé, Université Bordeaux 1&2, soutenue le 17 décembre 2010.

* Baptiste Guitton « Etude des déterminismes génétiques de l'alternance de production chez les pommiers », Ecole doctorale SIBAGHE, SupAgro (Montpellier), soutenue le 19 décembre 2011.

J'ai été (ou suis) membre de 11 comités de thèse

* Justine Perrotte (2011-2013) « Optimisation du potentiel de floraison chez le fraisier cultivé, *Fragaria* x *ananassa* : caractérisation moléculaire et fonctionnelle de gènes impliqués dans la floraison » (UMR Biologie du Fruit, INRA, Bordeaux).

* Youssef Chvan (2012-2015) « Bases génétiques de la croissance hétérotrophe chez *Medicago truncatula* ; impact du stress abiotique » (IRHS, Agrocampus Ouest, Angers).

* Mathieu Jourdan (2011-2013) « Impact de la variation nucléotidique de gènes de la voie de biosynthèse sur l'accumulation de caroténoïdes dans des ressources génétiques de carotte cultivée (*Daucus carota* L.) » (Agrocampus Ouest, Angers).

* Baptiste Guitton (208-2011) « Study of genetic determinisms of recurrent floral induction and its relation with architecture development in a perennial fruit tree species, the apple tree » (INRA, Montpellier)

* Saeïd Daghighi (2010) "Contribution de la cytogénétique à l'étude de la diversité génétique et à la caractérisation du gènome dans le genre *Rosa*" (UMR GenHort, Angers)

* Amélie Rabot (2007-2011) « Implication de la signalisation par les sucres dans la photomodulation du débourrement du bourgeon végétatif chez le rosier (UMR SAGAH, Angers)

* Amélia Gaston (2008-2011) « Identification et caractérisation du contrôle génétique et moléculaire de la 'remontée florale' chez le genre *Fragaria* (diploïde et octoploïde) » (UREF, Bordeaux)

* Amani Naouar (2007-2011) « Etude de gènes candidats pour la remontée de floraison par transgenèse et mise en place d'un protocole de transformation génétique du rosier » (UMR GenHort, Angers)

* Tiffanie Girault (2006-2009) "Etude du photocontrôle du débourrement chez le rosier" (UMR SAGAH, Angers)

* Jérémy Clotault (2006-2009) "Caractérisation de la diversité génétique chez Daucus carota par analyse de la variabilité de gènes de la voie de biosynthèse des caroténoïdes » (UMR GenHort, Angers)

* Virginie Boucher (2006-2009) "Analyse fonctionnelle de protéines de stress de type LEA (Late Embryogenic Abundant) impliquées dans la tolérance à la dessiccation et l'aptitude à la conservation chez la légumineuse modèle *Medicago truncatula* (UMR PMS, Angers)

J'ai participé à un concours de recrutement INRA pour un profil de TR « Biologie moléculaire » dans notre équipe. A l'automne 2012, je serai membre d'un jury de recrutement d'un maître de conférences à Agrocampus Ouest (site de Rennes).

5.5. Responsabilité collective

Au sein de l'UMR GenHort, j'étais animateur du groupe de biologie moléculaire dans le cadre de la démarche AQR (15 personnes). Ce groupe gère les activités collectives du groupe BM. J'ai ainsi supervisé la rénovation des laboratoires de biologie moléculaire (création d'un nouveau laboratoire dédié au génotypage), la mise en place d'un système de protection des échantillons à -80°C. Ce groupe gère un budget annuel de 20000€ (achat consommables et matériels). Au sein de l'IRHS, je continue à animer ce groupe pour le bâtiment B.

J'ai été animateur du comité éditorial de l'UMR GenHort. L'objectif de ce comité est de favoriser les publications de l'unité au niveau qualitatif et quantitatif (réalisation de groupe de travail sur une publication, analyse bibliométrique des publications de l'UMR). Ce comité n'a pas été maintenu au sein de l'IRHS. Entre 2006 à 2009, j'ai été membre élu du conseil de service de l'UMR GenHort.

Au niveau du centre INRA Angers-Nantes, entre 2007 et 2011, j'ai été membre du comité éditorial de la revue scientifique (Ad Litteram, 4 revues par an : <u>http://www.angers-nantes.inra.fr/le centre inra angers nantes/ad litteram lettre scientifique</u>).

Depuis 2011, je suis membre élu du conseil scientifique du département de génétique et amélioration des plantes de l'INRA. Je suis également membre de commission recherche de la CGT-INRA.

5.6. Liste des travaux encadrés

- Remay, A. (2009) Bases moléculaires de la floraison chez le rosier Thèse de doctorat de l'université d'Angers (soutenue le 17 septembre 2009).
- Emilie Araou (2011) Diversité génétique et origine de la remontée de floraison chez le rosier (Rapport de stage M2, Rech BioVigPa, Agrocampus Ouest, Centre d'Angers), Coencadrement avec Vanessa Soufflet-Freslon
- Youness Toualbia (2011) Etude fonctionnelle du gène de la remontée de floraison chez le rosier (Rapport de stage M2, Rech BioVigPa, Agrocampus Ouest, Centre d'Angers), Co-encadrement avec Marie Randoux
- Perrote, J. (2010) Contribution à l'étude du gène de la remontée de floraison chez le rosier (Rapport de stage M2 Rech BioVigPa, Agrocampus Ouest, Centre d'Angers), Coencadrement avec Laurence Hibrand-Saint Oyant.
- Vasseur, F. (2009) Etude du rôle des gènes de la voie des gibbérellines dans le contrôle de la remontée de floraison du rosier (Rapport de stage M2 Rech BioVigPa, Agrocampus Ouest, Centre de Rennes)
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6. Prospective : Projet de recherche

Le projet de recherche que je propose de mener s'appuie sur les résultats et les ressources que nous avons développés depuis plusieurs années sur le rosier. Il s'agira de **comprendre le rôle des gènes de la famille** *TFL1 / FT* **lors du développement du rosier par une approche pluridisciplinaire** en s'intéressant plus particulièrement à la floraison (date et remontée de floraison) et à l'architecture.

De plus, la réalisation de ce projet nécessite le développement d'outils de génomique. L'équipe a été impliquée dans le développement de différentes ressources (marqueurs génétiques, carte génétique, EST). Aujourd'hui un enjeu majeur pour la génomique du rosier est **l'obtention de la séquence du génome de rosier**, ressource nécessaire pour lancer des projets de génomique ambitieux, projets dans lesquels l'équipe que j'anime à toute sa place.

6.1. Rôle du gène RoKSN dans la saisonnalité de floraison chez le rosier.

6.1.1. La remontée de floraison est un caractère complexe.

La floraison a été principalement étudiée chez les plantes monocarpiques (telles que *Arabidopsis*, le maïs ou le pois). Récemment, différentes études ont été publiées concernant l'implication de différents gènes dans le contrôle de la floraison des plantes polycarpiques : *Arabis alpina* (Wang et al., 2009; Wang et al., 2011), *Lolium perenne* (MacMillan et al., 2005), la tomate (Pnueli et al., 1998; Lifschitz et al., 2006), le peuplier (Böhlenius et al., 2006; Hsu et al., 2006; Mohamed et al., 2010; Hsu et al., 2011) ou le fraisier (Koskela et al., 2012). Du fait des données et des ressources disponibles, le rosier pourrait être un modèle pour étudier la floraison chez les plantes polycarpiques, et plus particulièrement la saisonnalité de floraison.

Chez le rosier différents modes de floraison existent. Les rosiers sauvages sont principalement non remontants (NR), ils présentent une floraison uniquement au printemps. Cette floraison est sous le contrôle de facteurs environnementaux (froid, photopériode). De plus les rosiers NR présentent une phase juvénile.

Certains rosiers ont la capacité de fleurir plusieurs fois une même année, rosiers remontants (R). Parmi les rosiers remontants, on distingue les rosiers à floraison continue, qui fleurissent tant que les conditions environnementales sont favorables (figure 11a). La floraison continue est sous le contrôle d'un répresseur floral, *RoKSN*, homologue de *TFL1* (Iwata et al., 2012). Cette floraison continue s'explique par l'insertion d'un rétrotransposon dans le gène *RoKSN*. Cette mutation serait récessive et doit être à l'état homozygote pour que le caractère floraison continue s'exprime. Les rosiers à floraison continue seraient originaires de Chine et auraient été introduits en Europe au XVIII^{eme} siècle.

D'autres rosiers remontants (tels que certaines variétés de *R. damascena, R. moschata, R. rugosa* ou les mutants de type Climbing) refleurissent occasionnellement et faiblement principalement en automne (Figure 11a). Les mutants de type 'Climbing' présentent un allèle avec uniquement un élément LTR (Long Terminal Repeat) au niveau du gène KSN. Cet allèle serait dû à une recombinaison du rétro-transposon (Iwata et al., 2012). Cet allèle hypomorphe ne permettrait pas une restauration complète du phénotype. De plus, régulièrement nous

observons une faible remontée de floraison (deuxième floraison à l'automne) chez un hybride de *R. wichurana* qui est hétérozygote pour le gène *KSN* : un allèle sauvage et un allèle avec le rétro-transposon (résultats non publiés). L'allèle *copia* ne serait donc pas totalement récessif.

La remontée de floraison du rosier est donc un caractère quantitatif complexe. Nous avons mis en évidence un gène contrôlant la floraison continue (perte de fonction du gène *KSN*). De nombreuses questions persistent concernant la régulation de ce gène et son rôle dans le contrôle de la floraison, et du développement ainsi que son histoire au cours de la sélection du rosier, l'implication d'autres gènes dans la remontée de floraison. Le projet que je propose de développer a pour objectif de comprendre le rôle de *KSN* dans la floraison du rosier et plus largement lors du développement d'une plante polycarpique ligneuse en l'abordant par différentes approches:

6.1.2. Quelle est la régulation du gène RoKSN au cours de la saison par les facteurs internes et externes.

Chez les rosiers non-remontants, nous avons montré que *KSN* est réprimé au printemps (condition de floraison) et fortement exprimé plus tard (absence de floraison). Quels sont les facteurs (internes et environnementaux) qui contrôlent l'expression de *KSN* ?

→ Cette étude de la régulation de *KSN* passe par **une connaissance précise de l'expression spatio-temporelle du gène**. Pour cela, une étude sera réalisée par hybridation *in situ* (collaboration avec Annick Dubois, ENS Lyon) et complétée par une immuno-localisation de la protéine (travail de thèse de M. Randoux). En effet il a été montré chez *Arabidopsis*, que la protéine pouvaient migrer au sein du méristème (Conti and Bradley, 2007).

→ Importance de la voie des GA. Nous avons montré que le gène *RoKSN* est régulé au niveau transcriptionnel par les GA, et que cette régulation expliquait l'inhibition de la floraison par les GA au printemps chez les rosiers remontants (Figure 12, (Randoux et al., in press)). Cette étude sera poursuivie par : (i) analyse des éléments *cis* responsables de l'induction par les GA (caractérisation du promoteur), et (ii) caractérisation des éléments *trans* (simple hybride). Toutefois, la régulation de *RoKSN* par les GA ne représente surement pas l'unique régulation de *RoKSN*. Il semble que l'absence de refloraison ne puisse être uniquement expliquer par une régulation de *RoKSN* par les GA (Randoux et al., in press)

→ Importance des facteurs environnementaux (froid, photopériode). Les rosiers remontants sont auto-inductibles, ils n'ont pas besoin d'un contrôle environnemental pour fleurir, contrairement aux rosiers non-remontants qui sont sous contrôle de facteurs environnementaux (Havely, 1972; Foucher et al., 2008). La vernalisation serait nécessaire pour la floraison des rosiers non remontants. En effet « une vernalisation artificielle », en été, permet une refloraison des rosiers non remontant (résultats non publiés du laboratoire). Il s'agira de préciser l'effet de ce processus sur la floraison et sur l'expression du gène *RoKSN*. Une hypothèse qui sera testée est l'interaction possible entre les facteurs environnementaux et les GA. Il a été montré chez *Lolium perenne* que la perception des GA dépendait de la vernalisation (MacMillan et al., 2005).

6.1.3. Quel est le mode d'action du répresseur floral ?

Le mode d'action des répresseurs floraux de type *TFL1* est peu connu. Il a été montré que FT (FLOWERING LOCUS T), un activateur floral de la même famille que TFL1 (Bradley et al., 1997; Ratcliffe et al., 1998), est un régulateur d'un facteur de transcription de type β -ZIP, FD (FLOWERING LOCUS D). FT interagit avec FD et cette interaction permet

l'induction des gènes d'identité floral, *LEAFY* et *APETAL1* (Abe et al., 2005). TFL1 a une activité répressive qui nécessite la présence de FD (Hanano and Goto, 2011). Une hypothèse serait que TFL1 interagirait avec FD et empêcherait son activation (répression de la floraison). Ainsi l'activité antagoniste de FT et TFL1 pourrait s'expliquer par une compétition de FT et TFL1 vis-à-vis de FD (Ahn et al., 2006).

Chez le rosier, nous avons montré qu'au printemps l'apport exogène de GA bloque la floraison via l'induction de *RoKSN*. On observe toujours une induction de *RoFT*, mais une inhibition complète de *RoLFY* (Résultats Thèse Marie Randoux). Ainsi *RoKSN* bloquerait la floraison en aval de l'induction de *RoFT*. Une hypothèse intéressante pour expliquer cette inhibition serait la compétition de RoFT et RoKSN vis-à-vis de RoFD. Par double hybride, RoKSN et RoFT interagissent avec RoFD. Nous avons validé par la technique de FLIM-FRET une compétition entre RoKSN et RoFT vis-à-vis de RoFD (Travail de thèse de Marie Randoux, non publié). Il s'agit maintenant d'étudier l'effet de cette compétition sur la floraison. Pour cela, l'activation des cibles du complexe RoKSN/RoFT/RoFD est en cours d'étude.

6.1.4. Quelle est la fonction du gène *RoKSN* dans le contrôle de la floraison ?

La fonction du gène *RoKSN* sera validée par transgénèse chez le rosier. La validation sera réalisée par l'extinction du gène par RNAi chez des rosiers NR, qui devraient présenter une floraison continue. Comme aucun protocole de transformation génétique de rosier NR n'est disponible (Debener and Hibrand-Saint Oyant, 2009), un protocole est en cours de développement au sein de l'équipe. L'étude de ces plantes permettra de mieux comprendre la fonction de *RoKSN* dans le contrôle de la floraison et son implication dans d'autres processus de développement (voir 6.1.2).

6.1.5. D'autres gènes interviennent-il dans le contrôle de la remontée de floraison ? Existe-t-il un effet dose de l'allèle *RoKSN* sur le caractère quantitatif de la remontée de floraison.

Des facteurs génétiques pourraient également intervenir dans le contrôle de l'expression du gène et ainsi du caractère. En effet, certaines plantes hétérozygotes pour *RoKSN* (diploïdes ou tétraploïdes présentant l'allèle avec ou sans le rétransposon) présentent une remontée de floraison faible (données non publiées). Ce phénotype pourrait s'expliquer par d'autres loci contrôlant la remontée de floraison ou par un effet-dose au locus *RoKSN*. Le déterminisme génétique de la remontée de floraison sera abordé à partir de deux approches dans le cadre du projet SIFLOR. Nous rechercherons un lien entre le polymorphisme de gènes impliqués dans le contrôle de la floraison (dont *RoKSN*) et la remontée de floraison par une approche de génétique d'association sur une collection de rosier sélectionnée au XIX^{ème} siècle (échantillon de 150 cultivars). Cette approche devrait permettre également de valider par une autre approche l'implication de *RoKSN* dans le contrôle de la remontée de floraison. Cette collection sera également une ressource intéressante pour aborder l'effet dose du fait de la variation du niveau du ploïdie et du nombre de copies de l'allèle portant *copia*. Nous rechercherons une corrélation entre le nombre de copie de l'allèle *copia* et l'expression du phénotype.

De plus une descendance F2 diploïde, issus d'un croisement frère/sœur, sera analysée pour la recherche de QTL liés à la remontée de floraison. Cette population ségrégeant pour la remontée de floraison sera phénotypée et génotypée avec des SSR et des gènes de fonction connue impliquée dans le contrôle de la floraison. Le travail sur cette population permettra d'évaluer l'effet de dominance et l'effet dose des différents allèles au locus *RoKSN*. Cette étude sera complétée par une analyse de l'accumulation des transcrits *RoKSN* au sein des descendants.

6.1.6. Quelle est l'origine de la remontée de floraison chez le rosier ?

La floraison continue aurait été introduite au XVIII^{eme} siècle à partir de variétés cultivées chinoises, puis introgressée dans les rosiers européens pour donner naissance aux roses modernes (hybrides de Thé). Toutefois des rosiers faiblement remontants existaient déjà en Europe (type *R. damascena* « Quatre saisons » ou *R. moschata*) ou en Asie (*R. rugosa*). Ces rosiers peuvent refleurir une deuxième fois plus tardivement dans la saison. Il existerait ainsi plusieurs origines possibles. Cette approche sera menée avec les autres membres de l'équipe travaillant sur la diversité du rosier (principalement dans le cadre des projets SIFLOR et FLORHIGE).

→ Quelle est l'origine du gène de la remontée de floraison en Asie ? Nous avons montré que la floraison continue s'expliquait par l'insertion d'un rétrotransposon de type *copia* dans le gène *RoKSN*. A partir de rosiers cultivés anciens et sauvages chinois, nous étudierons la diversité du gène *KSN* (présence du rétro-transposon et séquençage exon / intron). Des collaborations ont été mise en place pour accéder à des collections de rosiers sauvages et cultivés chinois (Mikinori Ogisu et Hikaru Iwata). La structuration de cette collection sera évaluée à l'aide de microsatellites. De plus la datation de l'évènement d'insertion du rétrotransposon sera réalisée en séquençant les extrémités LTR (Long Terminal Repeat) du rétrotransposon. Les premiers résultats préliminaires (stage M2 Emilie Araou) montrent une origine chinoise. On retrouve un SNP toujours associé au rétrotransposon, sauf chez les roses sauvages chinoises (*R. chinensis* var spontanea), ce qui laisserait supposer que le rétrotransposon se serait inséré au sein de cette espèce.

→ Existe-t-il d'autres origines de la remontée de floraison ? L'expression du caractère remontée de floraison est variable selon les allèles (existence d'allèles hypomorphes, tels que l'allèle avec le LTR). L'analyse de la séquence de *KSN* chez *R. moschata* a permis de mettre en évidence des SNPs non synonymes pouvant affecter l'activité de la protéine (Stage M2, Emilie Araou). Ces nouveaux allèles d'origine européenne pourraient être responsables de la remontée observée. L'objectif sera de comprendre comment ces allèles nouvellement identifiés peuvent influencer la remontée de floraison : validation fonctionnelle (complémentation des mutants *tfl1* d'Arabidopsis), étude de l'expression du gène au cours de la floraison chez les génotypes, étude génétique (croisement *R. moschata* avec un rosier à floraison continue), importance de ces allèles au cours de la sélection.

6.1.7. Diversité et sélection du gène de la remontée de floraison.

La floraison continue aurait été sélectionnée en Chine. L'introduction se serait faite progressivement dans différents groupes horticoles (Noisette, Bourbon, Portland, Thé) pour arriver aux roses modernes remontantes (Hybride de Thé). Il s'agira d'étudier la diversité du gène *RoKSN* au cours de la sélection des roses anciennes européennes (*R. gallica, R. alba, R. damascena, R. centifolia*) et des différents groupes horticoles (Noisette, Bourbon, Portland, Thé et hybride de Thé) et de rechercher des traces de sélection au cours du processus de sélection (Autres approches développées dans le projet SIFLOR).

Ce projet d'étude de la sélection du gène de la remontée de floraison s'inscrit dans un projet pluridisciplinaire avec les historiens. Ce projet (FLORHIGE) vise à identifier les facteurs de réussite de l'innovation en horticulture ornementale par une démarche interdisciplinaire génétique et historique portant sur la sélection des rosiers aux XVIII^e et XIX^e siècles, dates clé de la sélection du rosier en Europe. Ce projet comprend un axe génétique qui vise à rechercher des signatures de sélection et identifier les bases génétiques d'intérêts (par une approche génétique d'association) sur une collection de rosiers anciens. Ce projet s'intéressera à la remontée de floraison, mais aussi aux autres caractères qui auraient pu être sélectionnés au cours de cette période. Un axe historique visera à retracer la complexité des processus, évènements, connaissances et contraintes qui ont influencé la création variétale en les replaçant dans le contexte de l'époque. Un autre axe analysera l'impact de la constitution des collections (roseraies) sur la création variétale et sur la conservation de la diversité des rosiers. Les données génétiques et historiques seront confrontées tout au long du projet pour aboutir à la constitution des échantillons génétique et à une compréhension des processus d'innovation. Ce projet (270 k€), que je coordonne, est financé par la région Pays de la Loire dans le cadre d'un appel d'offre 'paris scientifiques'. Il regroupe l'équipe GDO, des chercheurs du CERIO (Centre de Recherche en Histoire de l'Ouest, Université d'Angers / CNRS), du centre François Viète d'épistémologie et d'histoire des sciences et des techniques (Université de Nantes), l'équipe d'étude du polymorphisme des génomes végétaux (EPGV, INRA, Evry) et différentes roseraies : Roseraie Loubert (Rosier sur Loire, 49), Roseraie de la Cour de Commer (Commer, 53), Roseraie de la Beaujoire (Nantes, 44) et Roseraie du Val de Marne (Haÿ les Roses, 94).

6.2. KSN et le développement du rosier

6.2.1. Rôle des gènes *TFL1/FT* dans le développement des plantes.

Le rôle des gènes FT/TFL1 a été démontré pour de nombreuses espèces monocarpiques et polycarpiques dans le contrôle de la floraison en tant qu'activateurs et répresseurs floraux respectivement. Toutefois, il a été montré chez la betterave qu'un homologue de FT était un répresseur floral et intervenait lors de la vernalisation (Pin et al., 2010). De même chez le tabac, trois gènes similaires à FT ont une activité répressive (Harig et al., 2012). Récemment, chez Arabidopsis, le gène FT a été montré jouer un rôle dans la regulation de l'ouverture des stomates via l'activation d'une H+ ATPase (Kinoshita et al., 2011).

Les études réalisées chez les plantes polycarpiques ont permis de proposer de nouvelles fonctions, non plus uniquement dans le contrôle de la floraison, mais plus généralement au cours du développement de la plante. *TFL1/FT* contrôleraient la durée de la phase végétative chez le peuplier (Böhlenius et al., 2006) ou chez le pommier (Kotoda et al., 2010; Trankner et al., 2010). Chez le peuplier, un rôle a également été proposé au cours du débourrement (Rinne et al., 2011) et de l'entrée en dormance (Böhlenius et al., 2006; Ruonala et al., 2008). Chez la tomate, le ratio FT/TFL1 interviendrait dans le contrôle de différents processus du développement, les auteurs allant jusqu'à parler d'une nouvelle hormone végétale (Shalit et al., 2009). Dans la même famille, le gène *BFT* interviendrait lors de la germination chez le blé (Nakamura et al., 2011). Le mode d'action de cette famille de gènes est encore inconnu en dehors de la floraison et de l'interaction avec la protéine FD. Toutefois, des approches de double hybrides chez la tomate et le pommier ont permis de montrer l'interaction avec des facteurs de transcription qui pourraient réguler l'architecture de la

plante (Pnueli et al., 2001; Mimida et al., 2011), laissant supposer un rôle plus général de cette famille de gènes comme régulateur positif ou négatif de facteurs de transcription.

6.2.2. TFL1 / FT chez le rosier.

Chez le rosier, les gènes de la famille *TFL1/FT* pourraient également intervenir au cours du développement. Les mutants *ksn* (plante à floraison continue) n'ont pas de phase juvénile et leur architecture est fortement modifiée (Iwata et al., 2012). De plus, nous avons montré que des QTLs contrôlant des caractères architecturaux (Kawamura et al., 2011) et stage M2 de Hanae Roman) colocalisent avec des gènes de la famille *TFL1 / FT (KSN, RoFT et RoTFL1)*, ainsi que le gène *RoFD* (Figure 13). Les gènes de la famille *TFL1/FT* pourraient intervenir dans le contrôle de la floraison et dans le développement des plantes.

Je propose d'approfondir cette analyse pour mieux comprendre le rôle de cette famille de gènes dans le développement du rosier. Le séquençage du rosier permettra d'avoir une vision claire des gènes de cette famille. Une étude de leur expression dans différents tissus et au cours du développement permettra de préciser leur fonction possible. L'analyse du phénotype des plantes transgéniques (RNAi *RoKSN*) permettra de préciser la fonction de *RoKSN* au cours du développement de la plante. Par des approches génétiques et moléculaires, l'interaction / compétition entre ces différents gènes (et leurs différents allèles) sera étudiée ainsi que l'impact sur le développement des plantes.

De plus, *RoKSN* est sous le contrôle des GA. Des résultats préliminaires montrent une régulation possible du gène par les sucres. Chez le peuplier, les homologues de *TFL1/FT* interviendraient lors du débourrement et de l'entrée en dormance (Ruonala et al., 2008; Rinne et al., 2011). L'équipe ArchE de l'IRHS s'intéresse au débourrement chez le rosier. Ils ont montré l'importance de la lumière et des sucres dans ce processus. Récemment, en collaboration, nous avons abordé le rôle des GA dans ce processus (Choubane et al., 2012). Il serait intéressant de voir l'implication possible de *RoKSN* (ou de son paralogue *RoTFL1*) dans ce processus et de son contrôle par les GA et les sucres.

6.3. Le séquençage du génome de la rose

Je me suis engagé activement depuis plusieurs années pour promouvoir la génomique du rosier. Cette action a été menée au niveau national (Groupe National Rose avec le développement de différentes ressources : EST, puce Affymetrix) et au niveau international (Organisation 1^{er} réunion sur la génomique du rosier, membre du consortium international pour le séquençage du rosier). Aujourd'hui Angers est reconnu internationalement pour ses études et ressources sur la génétique et la génomique du rosier. Ainsi dans le cadre du consortium international, les équipes françaises sont leaders pour le séquençage du génome. Le projet est porté par Mohammed Bendhamane (ENS, Lyon) et moi-même. En lien avec le consortium, une stratégie a été définie pour le séquençage.

6.3.1. Choix d'un génotype de référence : 'Old Blush'

Le génotype 'Old Blush' a été choisi comme génotype de référence par la communauté international. Il s'agit d'un génotype diploïde sur lequel de nombreuses équipes (dont l'ensemble des équipes françaises) travaillent. Différentes ressources ont été

développées sur cette variété. Nous avons participé dans le cadre d'un projet national à la production d'EST (résultats en cours de publication). L'équipe lyonnaise a développé un protocole de transformation (Vergne et al., 2010). De plus ce génotype a joué un rôle important dans la sélection du rosier (introduction de la remontée de floraison et du parfum rose de Thé). Toutefois ce rosier (comme la plupart des roses) est fortement hétérozygote. L'hétérozygotie a été estimée à plus de 80% à l'aide marqueurs microsatellites (D. Byrne, communication personnelle). Ainsi l'un des challenges concernant le séquençage du génome de rosier sera de résoudre ce problème d'hétérozygotie en combinant des approches de séquençage et de cartographie.

6.3.2. Obtention d'une carte génétique haute densité pour ancrer la séquence.

Cette carte sera développée à partir du matériel que nous avons produit à Angers (croisement entre 'Old Blush' et *R. x wichurana*). Cette descendance comprend plus de 300 individus. La carte « haute densité » sera développée sur 96 descendants. Cette ressource permettra d'ancrer rapidement le génome sur une carte génétique. A partir de cette carte haute densité, l'objectif est de sélectionner 10 génotypes qui maximisent les évènements de recombinaison (stratégie de bin-mapping) et de séquencer en profondeur ces 10 individus. Cette technique devrait permettre de résoudre les problèmes liés à l'hétérozygotie, de résoudre les haplotypes et de faciliter l'assemblage (si besoin).

De plus il s'agira d'une population de référence pour les études en génétique sur le rosier. Elle a ainsi été choisie dans le cadre d'un projet européen (Rosa2Genome, International Training Network) comme population de travail par une dizaine de laboratoires européens pour étudier le déterminisme génétique de nombreux caractères (floraison, architecture, stress biotiques et abiotiques). Ce projet ITN est en cours d'évaluation.

6.3.3. Développement d'haploïdes de 'Old Blush'

En parallèle de l'approche génétique, nous avons décidé de développer deux techniques différentes pour obtenir un haploïde de 'Old Blush'. Cet haploïde pourrait être une solution pour résoudre les problèmes d'assemblage lié à l'hétérozygotie de 'Old Blush'. Nous avons choisi d'utiliser une technique mise au point par l'INRA de Fréjus. Les haploïdes sont obtenus par parthénogenèse induite en utilisant du pollen irradié (Meynet et al., 1994). En parallèle, l'obtention d'haploïdes sera tentée à partir de culture de micropores de rosier suivi de régénération. Ces approches longues et incertaines sont en cours et pourraient permettre de résoudre les obstacles rencontrés.

6.3.4. Séquençage et assemblage de 'Old Blush'

Le séquençage est en cours de réalisation par le Génoscope qui réalisera un premièr assemblage (résultat attendu fin 2012). En fonction des difficultés rencontrées, il pourra s'appuyer sur la carte haute densité (voir point précédent). L'annotation sera réalisée par l'URGI (Versailles) et le LIPM (Toulouse). Pour l'annotation nous pourrons nous appuyer sur les EST que nous avons développés précédemment.

Le séquençage du génome permettra de faire progresser les connaissances dans différents domaines comme l'identification des bases moléculaires de caractères ornementaux importants, l'analyse de la diversité et de l'évolution des génomes ou la génomique comparative (au sein de la famille des *Rosaceae*). Il permettra de développer de nouveaux outils pour faciliter le génotypage (puce ADN) ou l'étude du transcriptome (puce, RNA Seq).

La recherche de gène-candidat colocalisant avec les QTL sera facilitée. Dans le cadre du projet ITN (Rosa2Genome), un effort important a été réalisé pour associer les entreprises privées au projet et mettre en place des formations pour permettre à ces entreprises d'intégrer les retombées issues du séquençage dans leur programme de sélection.

7. Réflexion générale sur le diplôme de « l'Habilitation à Diriger des Recherche » (HDR).

Un peu d'histoire...

L'habilitation à diriger des recherches (HDR) a été créée en application de la loi du 26 janvier 1984 sur l'enseignement supérieur. Cette loi a été modifiée par un arrêté ministériel du 23 novembre 1988. Cet arrêté donne le cadre de l'HDR et de sa délivrance. Il a été explicité par une circulaire (89-004) du 5 janvier 1989 (RLR 430-5). Il n'est pas clair dans les textes, mais il semble que l'HDR ait été créé pour succéder au doctorat d'Etat.

Qu'est ce que l'HDR ?

L'article 1 stipule : « L'habilitation à diriger des recherche sanctionne la reconnaissance du haut niveau scientifique du candidat, du caractère original de sa démarche dans un domaine de la science, de son aptitude à maîtriser une activité de recherche dans un domaine scientifique ou technologique suffisamment large et de sa capacité à encadrer des jeunes chercheurs. Elle permet notamment d'être candidat à l'accès au corps des professeurs des universités ». La circulaire précise « L'HDR est un diplôme dont la finalité essentielle, sinon exclusive, est de permettre l'accès au corps des professeurs des universités ». Il est à noter que dans certaines disciplines (juridiques, politiques, économiques et de gestion) il existe certaines dispositions pour que la soutenance de la thèse et de l'HDR aient lieu en même temps.

Y-a-t-il un équivalent ailleurs de l'HDR ?

Alors que dans le système anglo-saxon la thèse est le diplôme universitaire le plus élevé, on retrouve en Europe un système qui se rapproche de l'HDR, il s'agit du '*Privatdozent*' (*PD*, que l'on peut traduite par 'enseignant privé'). *PD* est un titre de tradition allemande et qui permet aux personnes qui n'ont pas de poste (de chaire) de pouvoir enseigner à l'université sans être payé. C'est souvent un titre nécessaire pour avoir un poste (source Wikipédia). Ce système se retrouve dans de nombreux pays européens. La mise en place de ce statut de 'Privatdozent' au XIXe siècle allait créer un « prolétariat académique » selon les termes de E. Durkheim, « une forme de misère de statut, doublée d'une misère matérielle considérable, au moins en ce qui concerne les « Privatdozenten » issus de milieux sociaux non privilégiés (Busch, 1963; Schultheis, 2000). Certains s'interrogent après la défaite de 1870 de l'importance de ce système dans la réussite des universités allemandes et de façon générale du succès de l'Allemagne. Ce système existe encore en Europe. On le retrouve ainsi dans des universités suisses. Il permet d'enseigner à l'université sans avoir de poste, mais maintenant avec un salaire !

L'HDR en débat ?

Aujourd'hui, l'opportunité de maintenir ce système en France se pose. On remarque que l'HDR existe principalement pour l'accès au corps de professeur de l'université et de l'enseignement supérieur agronomique. Ce système français, souvent inconnu des étrangers ou peu accessible aux expatriés, est donc une limite à l'intégration de certaines personnes aux corps des professeurs. De plus on peut s'interroger sur le doublon que sont l'HDR et les qualifications par le CNU (Comité National des Universités). Ne serait-il pas plus simple de maintenir uniquement le système des qualifications ? Un rapport d'un sénateur écrivait même sur l'HDR « les commissions de spécialistes lorsqu'elles doivent procéder au recrutement d'un professeur, examinent la liste de ses travaux, et y trouvent des critères d'évaluation qui rendent superflu le passage de l'HDR ».

L'HDR est l'occasion de faire le point sur son activité passée et future. En ce qui concerne les chercheurs de l'INRA, ce travail a lieu régulièrement dans le cadre des évaluations des chercheurs par les CSS (Commissions de Spécialistes Scientifiques). Une évaluation approfondie a lieu tous les 2 ans pour les CR2 et tous les 4 ans pour les CR1. Cette évaluation est aussi l'occasion de faire le point et d'avoir un avis sur son projet et sur son orientation.

De plus n'y-t-il pas une contradiction à devoir avoir l'HDR pour encadrer un doctorant, et devoir avoir déjà encadré un doctorant pour soutenir son HDR. En quoi un jeune CR ou maître de conférences est-il moins à même d'encadrer un doctorant ? De plus, aujourd'hui des garde-fous existent comme les comités de thèse qui permettent (parfois à minima) de palier des encadrements déficients, et pas nécessairement des encadrements par des jeunes chercheurs sans HDR. De même, est-on vraiment plus sûr qu'un encadrement par un chercheur avec une HDR se passsera mieux que sans ?

Concernant l'encadrement, on peut noter une proposition issue des réflexions qui ont eu lieu dans le monde de la recherche en 2003 et qui ont débouché sur les assises de Grenoble. Il s'agit de la mise en place d'une HDR à point, soit l'HPR, « l'habilitation à point à diriger des recherches ». On obtient 2 points dès que l'on obtient un poste permanent, et l'on peut perdre des points suite à des problèmes d'encadrement de thèse. Le détail de la proposition est en ligne (http://fgimello.free.fr/CV-Bio-Liens/HPR.htm). Cette proposition originale pourrait être rediscutée lors des assises de la recherche qui ont lieu actuellement.

L'HDR est nécessaire pour devenir Professeur des Universités. Si nous reprenons les termes de l'article 1, l'HDR « reconnait le haut niveau scientifique du candidat », il n'y est pas fait référence aux qualités d'enseignant. En Allemagne, jusqu'en 2002 pour devenir professeur, enseignement et recherche étaient pris en compte : le travail scientifique via une habilitation ('Kumulative Habilitation') et la capacité à enseigner (évaluer par les cours à l'université et l'encadrement des étudiants). Si le candidat n'a pas d'expérience en enseignement, il doit faire des cours devant des étudiants et des membres de l'université. Ce système pourrait globalement être comparé aux qualifications françaises. Ensuite le candidat présente un sujet devant un comité (Habilitationsvortrag). Après délibération, le comité donne l'autorisation d'enseigner et la discipline (venia legendi), il donne également l'habilitation pour la recherche. Depuis 2002, l'habilitation n'est plus nécessaire, mais les jeunes chercheurs continuent majoritairement à la passer (Source : Pr Thomas Debener, University of Hannover).

L'ensemble de ces éléments dressent un tableau assez confus concernant l'HDR et sa place dans le système de recherche et d'enseignement français (voire européen). On peut légitiment s'interroger aujourd'hui sur le maintien de ce diplôme. Il est l'occasion de faire un point sur sa carrière et d'avoir un regard critique sur son travail ; finalement peut-être est-ce déjà suffisant ?

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Annexe 1 : Bilan des projets déposés depuis 2003 (date de mon recrutement à l'INRA).

Titre	Acronyme	Durée	Somme	Port	date	Guichet (3)	Résultats
			(1)	/Part(2)			(4)
Séquençage du génome de la rose.	ROSA_GEN	24	112	Port	2012	Region Pays de	С
	OME					la Loire	
GENOMICS2ROSES-ITN	GENOMICS	36		Part	2012	FP7, Marie-	С
	2ROSES					Curie, ITN	
PLATEFORME REGIONALE D'INNOVATION : INNOVATION ET		48	70	Assoc	2012	Région Pays de	А
MODERNISATION DE LA FILIERE PEPINIERE ET ROSIER DU BASSIN DOUESSIN						la Loire	
Etude de la diversité de Dilocarpon rosae agent de la maladie des taches noires du rosier	DIRO	24	7	Port	2012	SFR Qusav	Α
Facteurs de succès de l'innovation variétale du rosier aux XVIII et XIXe	FloRHiGe	36	209	Port	2012	Région	А
siècles en France : démarche interdisciplinaire génétique et historique							
Signature de sélection et déterminisme génétique de la remontée de floraison chez le rosier	SIFLOR	36	30,5	Port	2012	INRA-DGAP	Α
The genome of the woody ornamental Rosa sp	RosaGenom	12	30	Assoc	2011	INRA-AIP	Α
Identification et caractérisation des mutations DOMINANT DOUBLE (DODO) an DOUBLE FLOWER (DF) chez le pétunia et la rose	dodo	36	480	Assoc	2012	ANR blanc	R
Translating knowledge of the control of flowering time and duration to improve breeding efficiency and to realize cropping potential in a model family of perennial plants, the Rosaceae	PerflowEU	36	158(a)	Assoc	2011	FP6 - KBBE	R
Genome engineering improvement for usefull plants of a sustainable	Genius			Assoc	2010	Investissement	R
agriculture					2011	d'avenir (ANR)	Α
Maîtrise des maladies fongiques du feuillage des rosiers de jardin	Rosa	36	300	Assoc	2010	CASDAR	Α
	fortissima						

Contrôle de la floraison continue chez les plantes pérennes.	Rebloom	48	268	Port	2010	ANR Blanche	R
Validation fonctionnelle du gène de la remontée de floraison	refloraison	18	75	Port	2010	INRA - Prévalorisation	R
Développement d'outils pour la génomique chez la rose :Identification de gènes associés aux caractères d'intérêt.	RosaGen	48	891	Assoc	2010	ANR	R
Transformation Génétique des Rosacées	TGR	36		Port	2010	INRA-DGAP	R
Développement d'outils pour la génomique chez la rose :Identification de gènes associés aux caractères d'intérêt	RosaGen	36	978	Assoc	2009	ANR Génomique	R
Modélisation de la plasticité architecturale des plantes en fonction de facteurs génétiques et en réponse à des stress environnementaux	PhenoGeneA rch	24	134	Assoc	2009 2010	ARC	R R
Transcriptome du rosier : Obtention et séquençage d'une population d'EST représentant les gènes exprimés chez le rosier	RosaEST	12	30	Assoc	2008	INRA- AIP Bioressources	A
Développement de méthodes pour la quantification allélique et application		12	11,8	Assoc	2008	INRA DGAP	А
Développement de techniques de transformation génétique du rosier		36	195	Port	2007	CTPS	А
La floraison chez les Rosoideae (<i>Fragaria</i> and <i>Rosa</i>): contrôle génétique et moléculaire de la remontée florale	ROSOFLO W	36	95(a)	Assoc	2007	ANR Génomique	R
Flowering initiation and development in the Rosaceae family using an integrated approach: from comparative genomic to breeding	ROSAFLOR	36		Assoc	2007	FP7, Marie- Curie, ITN	R
Demande Post-Doc concernant Jean-Michel Davière.	Rosa-GA	24		Port	2007	FP7, People, IEF	R
Importance des gènes de ramification (<i>MAX/RMS</i>) et de la voie des gibbérellines dans l'édification architecturale de deux systèmes végétaux ligneux : Pommier et Rosier		36	41	Assoc	2007	INRA DGAP	R

Identification d'un panel de SNP dans des gènes de fonction connue le long	RosaSNP	18	6,6	Port	2007	INRA DGAP -	А
du génome chez le rosier						Génotypage	
Construction et Santé du Végétal	COSAVE	48		Assoc	2006	Région	А
Etude de la variabilité fonctionnelle chez le rosier : faisabilité de	VAROFON	12	9.5	Assoc	2006	INRA-DGAP	А
la technique de pyroséquençage							
Comparative mapping towards functional synteny analyses in Rosaceae: the	RoSyn	48		Assoc	2005	FP6- Mobility	R
fore runner to innovative fruit and flowers.							
Recherche de SNP ou de InDel dans des gènes impliqués dans le		36	300	Assoc	2003	GENOPLANTE	R
déterminisme de la date de floraison chez les dicotylédones cultivées							

(1) Somme : somme en k€ demandée dans le projet

(2) Port/Part : Port : lorsque j'ai été porteur du projet ; Part : lorsque j'ai été partenaire du projet

(3) Différentes agences auprès desquelles le projet a été soumis : ANR (Agence Nationale pour la Recherche), Région (Appel d'offre de la région Pays de la Loire), CTPS (Comité Technique Permanent de la Sélection), IEF (Intra European Fellowship for Career Development).

(4) Résultats suite à l'évaluation des projets : A : accepté, R : Refusé, C : en cours.

(a) La somme correspond à la somme demandée par l'équipe et non le coût total du projet.

Annexe 2 : Les cinq publications jugées les plus significatives de ma carrière

- Randoux, M, Jeauffre, J, Thouroude, T, Vasseur, F, Hamama, L, Juchaux, M, Sakr, S, and Foucher, F. in press. The continuous flowering regulator, RoKSN, a TFL1 homologue, mediates inhibition of flowering by gibberellins in once-flowering roses. J Exp Bot.
- Iwata, H, Gaston, A, Remay, A, Thouroude, T, Jeauffre, J, Kawamura, K, Hibrand-Saint Oyant, L, Araki, T, Denoyes, B, and Foucher, F. 2012. The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. The Plant Journal 69, 116-125.
- **Dubois, A, Remay, A, Raymond, O, et al.** 2011. Genomic Approach to Study Floral Development Genes in *Rosa sp.* PLoS ONE **6**, e28455.
- Remay, A, Lalanne, D, Thouroude, T, Le Couviour, F, Hibrand-Saint Oyant, L, and Foucher, F. 2009. A survey of flowering genes reveals the role of gibberellins in floral control in rose. Theor Appl Genet 119, 767-781.
- Foucher, F, Morin, J, Courtiade, J, Cadioux, S, Ellis, N, Banfield, MJ, and Rameau, C. 2003. DETERMINATE and LATE FLOWERING are two TERMINAL FLOWER1/CENTRORADIALIS homologs that control two distinct phases of flowering initiation and development in Pea. Plant Cell 15, 2742-2754.

RESEARCH PAPER

Gibberellins regulate the transcription of the continuous flowering regulator, *RoKSN*, a rose *TFL1* homologue

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Abstract

The role of gibberellins (GAs) during floral induction has been widely studied in the annual plant *Arabidopsis thaliana*. Less is known about this control in perennials. It is thought that GA is a major regulator of flowering in rose. In spring, low GA content may be necessary for floral initiation. GA inhibited flowering in once-flowering roses, whereas GA did not block blooming in continuous-flowering roses. Recently, *RoKSN*, a homologue of *TFL1*, was shown to control continuous flowering. The loss of *RoKSN* function led to continuous flowering behaviour. The objective of this study was to understand the molecular control of flowering by GA and the involvement of *RoKSN* in this inhibition. In once-flowering rose, the exogenous application of GA₃ in spring inhibited floral initiation. Application of GA₃ during a short period of 1 month, corresponding to the floral transition, was sufficient to inhibit flowering. At the molecular level, *RoKSN* transcripts were accumulated after GA₃ treatment. In spring, this accumulation is correlated with floral inhibition. Other floral genes such as *RoFT*, *RoSOC1*, and *RoAP1* were repressed in a *RoKSN*-dependent pathway, whereas *RoLFY* and *RoFD* repression was *RoKSN* independent. The *RoKSN* promoter contained GA-responsive *cis*-elements, whose deletion suppressed the response to GA in a heterologous system. In summer, once-flowering roses did not flower even after exogenous application of a GA synthesis inhibitor that failed to repress *RoKSN*. A model is presented for the GA inhibition of flowering in spring mediated by the induction of *RoKSN*. In summer, factors other than GA may control *RoKSN*.

Key words: Floral initiation, floral repressor, gibberellins, PEBP family, polycarpic plants, rose.

Introduction

Gibberellins (GAs), important plant growth regulators, are involved in different aspects of plant development such as growth promotion, seed dormancy, floral initiation, and development (Fleet and Sun, 2005). GAs are cyclic diterpenoid molecules, with GA₁, GA₃, GA₄, and GA₇ being the most biologically active in plants. The floral transition from vegetative growth to flowering is an important period in the life of a plant. In longday (LD) monocarpic plants, it was reported that GA is a floral stimulus and could be substituted for LDs (Lang, 1965) whereas, in most woody plants, GA inhibits flowering (Zeevaart, 1976).



Abbreviations: CF, continuous flowering; DAB, days after the beginning of treatment; GA, gibberellin; OF, once-flowering; PCB, paclobutrazol. © 2012 The Author(s).

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For example, GA activates flowering in *Arabidopsis thaliana*, whereas it represses flowering in woody fruit trees (Wilkie *et al.*, 2008).

The molecular basis of floral transition and, particularly, the response to GA, was elucidated in the model plant *A. thaliana*, a facultative LD vernalization-mediated plant. For this transition to be successful, *Arabidopsis* must integrate different endogenous and environmental signals (reviewed by Srikanth and Smith, 2011). Flowering is controlled by four major genetic pathways: photoperiod, autonomous, GA, and vernalization. These different pathways converge to activate the floral integrators, *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF CONSTANS1 (SOC1)*, and to repress *FLOWERING LOCUS C (FLC)*, a floral repressor involved in the vernalization response (Kim *et al.*, 2009). The floral integrators then activate the meristem identity genes, *LEAFY (LFY)* and *APETALA1 (AP1)*, leading to activation of the floral organ identity genes known as ABCE model genes.

In *Arabidopsis*, GA was required for flowering under short days (SDs) and LDs, with a weaker effect under LDs. *ga1-3*, a GA-deficient mutant due to a mutation in a key GA metabolism gene (Koornneef and van der Veen, 1980), led to a delay in flowering under LDs and was unable to flower under SDs. This phenotype was reversed by exogenous application of GA_4 (Wilson *et al.*, 1992). Under SDs, GA_4 was accumulated in the shoot apical meristem (SAM) prior to floral transition. GA_4 may be synthesized in the leaves and then move to the apex (Eriksson *et al.*, 2006).

GAs induce flowering through two different pathways. In leaves, the GA response was FT dependent. Under LDs, GA activated CONSTANS (CO) that in turn induced FT (Hisamatsu and King, 2008). In the apex, GA acted in an FT-independent manner to induce SOC1 and LFY (Hisamatsu and King, 2008). Failure of the gal-3 mutant to flower under SDs was due to the absence of up-regulation of the floral integrator SOC1 (Moon et al., 2003) and the meristem identity gene LFY (Blazquez et al., 1998). In gal-3 mutants, SOC1 was accumulated after exogenous GA_4 application under SDs (Moon et al., 2003). GA₄ regulation of SOC1 expression could be mediated by the down-regulation of SHORT VEGETATIVE PHASE (SVP), a floral repressor involved in the vernalization and ambient temperature pathways (Li et al., 2008). The GA effect on LFY expression is mediated by a transcription factor, AtMYB33, which is able to link the GAMYBbinding site in the LFY promoter (Gocal et al., 2001). AtMYB33 expression corresponds to GA₄ accumulation in the shoot apex during floral transition (Gocal et al., 2001; Eriksson et al., 2006).

Except for *Arabidopsis*, few data were available about the molecular control of flowering by GAs in other species. In *Lolium temulentum*, an obligate LD plant, GA activated flowering (Evans *et al.*, 1990). The leaf GA content increased after LD exposure with transcriptional regulation of GA biosynthesis genes (Gocal *et al.*, 1999). GA moves from leaves to the shoot apex where the increase in GA content corresponds to floral induction (King *et al.*, 2001). LD treatment can be mimicked by GA application, especially GA₅ and GA₆ (Evans *et al.*, 1990; King *et al.*, 2001). *Lolium perenne*, a perennial near-relative of *L. temulentum*, requires both LDs and vernalization for flowering. LDs activate GA biosynthesis, which then stimulates the flowering of vernalized plants, whereas non-vernalized plants are unable to respond

to GA for flowering (MacMillan *et al.*, 2005). In grapevine, the *vvGAI* mutant, a gene encoding a DELLA protein, a central repressor of GA signalling, was dwarf, GA-insensitive, and produced inflorescences along the length of the shoot instead of tendrils. GA represses the formation of inflorescences and promotes the initiation of tendrils (Boss and Thomas, 2002).

Rose is an economically important ornamental crop worldwide. Flowers represent the major value of roses, and control of flowering is a major issue. Rose is a perennial woody plant with different modes of flowering. Once-flowering (OF) roses have a single annual flowering period, whereas continuous-flowering (CF) roses have the ability to flower continuously during the season. In rose, the sensitivity of plants to GA was different between OF and CF roses. Exogenous application of GA inhibited flowering in OF roses and had no effect on flowering in CF roses (Roberts et al., 1999). GA signalling genes such as GID1, a GA receptor, are regulated differently between OF and CF roses (Remay et al., 2009). Furthermore, GA metabolism was involved in floral initiation. In OF and CF roses, the amount of GA in shoots before floral initiation was low and increased after floral initiation (Roberts et al., 1999). This GA content was correlated with GA metabolism gene regulation during floral transition. In OF and CF roses, the RoGA20ox gene, encoding an enzyme involved in GA synthesis, was transiently repressed before floral initiation. In contrast, the expression of *RoGA2ox*, encoding an enzyme involved in GA inactivation, increased before floral initiation and decreased later (Remay et al., 2009). Recently, the gene encoding the continuous flowering locus was shown to correspond to a floral repressor homologue of TFL1, TERMINAL FLOWER1, and was designated as RoKSN (Iwata et al., 2012). CF roses had a retrotransposon inserted in the second intron of *RoKSN*. The presence of a retrotransposon blocked the transcription of RoKSN in CF roses, allowing continuous blooming. In OF roses, RoKSN is weakly accumulated in spring to permit blooming. Later in the season, RoKSN transcripts accumulate and shoots remain vegetative (Iwata et al., 2012).

The objectives of this study were to understand the molecular control of flowering by GA in rose and to decipher the link between the continuous flowering gene, *RoKSN*, a TFL1 homologue, and GA. In this study, it was demonstrated that GA inhibited floral initiation in rose by up-regulating the CF gene at the transcriptional level. The promoter of RoKSN was further analysed, and GA-responsive elements were found.

Materials and methods

Plant material

Rosa×wichurana (RW), an OF rose, was obtained from the rose garden, Jardin de Bagatelle (Paris, France). The mutant pair, *R. hybrida* 'Félicité et Perpétue' (FP), an OF rose, and 'Little White Pet' (LWP), its CF mutant, were obtained from 'Loubert Nursery' (Rosier sur Loire, France). Plants were cultivated as described (Remay *et al.*, 2009). *Nicotiana benthamiana* were maintained in a greenhouse (20 °C, 16h light), and 6- to 8-week-old plants were used for agroinfiltration.

GA and paclobutrazol treatments on plants

In December, stems were pruned at six internodes and maintained outside for vernalization (Fig. 1A). At the beginning of the treatment (end of January), plants were placed in a tunnel. Different GA₃ concentrations (0, 30, 70, and 140 μ M) were sprayed on plants three times a week during different periods and for different durations (Fig. 1B). Paclobutrazol (PCB) treatment (Bonzi[®], at 42, 70, or 112 μ M) was performed every 2 weeks. After blooming, a new set of plants was used for GA₃ and PCB treatments. The inflorescences were removed and growing shoots were pruned at six nodes. Plants, maintained in a greenhouse, were sprayed with GA₃ (30 μ M) three times a week for 34 d and with PCB (Bonzi[®], at 14, 56, or 112 μ M) three times during the experiment [0, 15, and 29 days after the beginning of treatment (DAB)].

Phenotypic observations

Phenotypic observations were carried out on 10 plants with approximately five pruned stems, 2 months after the end of the treatment. Data were gathered from the three new emerging shoots placed on the distal part of the pruned stem (Fig. 1A). For each new emerging shoot, the percentage of flowering that represents the ratio between the number of flowering shoots and the number of shoots was determined. To calculate the average internode size, three consecutive internodes placed on the apical part of the stem were measured. Statistical analyses were done using the R software, version 2.13.1.

Histological observations

After dissection (removal of leaves), samples were fixed and sections of $2-3 \mu m$ were observed under a microscope (Foucher *et al.*, 2008). Observations were made on four different dates (42, 56, 63, and 70 DAB).

RNA isolation and gene expression analysis

For RNA isolation, the terminal part of the shoot was harvested from five plants per date and per treatment (Fig. 1A). Samples were dissected (removal of expanded leaves and young leaves) and frozen in liquid nitrogen. Total RNA was isolated using the NucleoSpin[®] RNA plant kit (Macherey-Nagel) according to the manufacturer's instructions.



Fig. 1. Management of *R.×wichurana* (RW) after GA₃ and PCB treatments. (A) In December, vegetative shoots were pruned at six nodes. Plants remained outside for vernalization. At the end of January, plants were transferred to a tunnel. New shoots developed from axillary buds of the previous years. These shoots were mainly terminated by an inflorescence. After floral development, new shoots grew from the base. Since RW is an OF rose, the shoots remained vegetative. In summer, these shoots were pruned at six nodes and the fate of new emerging shoots from axillary buds was studied. Under controlled conditions, these shoots remained vegetative. RW plants were treated with GA₃ (0, 30, 70, or 140 μ M) during vegetative growth and floral initiation between January and March (hatched boxes). Another set of RWs was treated with GA₃ (30 μ M) and PCB (14, 56, and 112 μ M) during vegetative growth between August and September. Arrows and asterisks indicate the sampling carried out for RNA extraction and histological analysis, respectively. (B) Experiments with different durations and periods of GA₃ (30 μ M) treatment. In the 'Late treatment', the experiment started at different times and ended at the same time, whereas in the 'Early treatment', the experiment started at the same time and ended at different times. Numbers indicate the days after the beginning of treatment (DAB). Broken lines represent the pruned shoots of the previous years, large arrows represent new indeterminate growing shoots, and black circles represent axillary buds. Open circles represent flowers. (This figure is available in colour at *JXB* online.)

Elimination of genomic DNA and reverse transcription were performed using 1 mg of total RNA; real-time PCRs were performed using 3 μ l of reverse transcription product (1/25 dilution), as described by Remay *et al.* (2009). The amount of plant RNA in each sample was normalized using *TCTP* and *UBC* genes (Klie and Debener, 2011), and the relative expression level was calculated according to Pfaffl (2001), from two biological replicates and three technical repetitions per replicate.

DNA constructs

The upstream genomic sequence of RoKSN (promoter region) was obtained using the Genome Walker Universal Kit (Clontech), following the manufacturer's recommendations from genomic DNA libraries of R. hybrida 'Knock Out'. Serial nested PCRs were performed using the described reverse primers (Supplementary Table S1 available at JXB online) and the forward primers of the kit. A 1200 bp fragment was amplified and sequenced. Then, using Finnzymes' Phusion® High fidelity DNA Polymerase (Thermo Fisher Scientific), the promoter region was amplified from DNA of Rosa chinensis 'Old Blush' and cloned into the pGEM T easy vector (Promega). Next, successive promoter deletions were performed to obtain fragments of different sizes: 200, 400, 800, and 1200 bp. PCR products were cloned using the Gateway[®] system (Invitrogen) with TOPO isomerase mix into the pENTRTM/D-TOPO[®] entry, followed by LR clonase recombination with pKGWFS7 (VIB, Gent, Belgium) (Karimi et al., 2005). The promoter sequence was fused to a GUS::GFP (β-glucuronidase::green fluorescent protein)coding gene. Constructions were introduced by electroporation in Agrobacterium tumefaciens EHA105 with the plasmid pbbR.

Transient expression procedure

Agrobacterium tumefaciens C58C1 containing p19 (Voinnet *et al.*, 2003) and EHA105 containing the different constructions were grown at 28 °C in LB medium supplemented with 50 mg l⁻¹ kanamycin, 50 mg l⁻¹ rifampicin, and 100 mg l⁻¹ gentamicin (only for EHA105) up to the stationary phase. After centrifugation at 5000 g for 10 min, bacteria were resuspended in Murashige and Skoog (MS) medium supplemented with 5% (w/v) sucrose, acetosyringone (200 μ M), pH 5.6, and with or without GA₃ (10 μ M) to obtain an optical density (OD₆₀₀) of 0.4. Two days after agroinfiltration of 6- to 8-week-old *N. benthamiana* leaves, GFP fluorescence was observed using a Nikon A1 laser scanning confocal microscope.

Sequence analysis

Sequences of promoters of *RoKSN* (HE863824), *MdTFL1*, *MdTFL1a* (AB366639 and AB36640), *FvKSN* (HQ378595), *PpTFL1* (AB636121), and *TFL1* (At5g03837) were used to perform the alignment with MVISTA online software (http://genome.lbl.gov/vista/mvista/submit. shtml) (Frazer *et al.*, 2004). The search for putative binding sites of *cis*-elements was then carried out using Plantpan online software (http:// plantpan.mbc.nctu.edu.tw/seq_analysis.php) (Chang *et al.*, 2008).

GUS activity

To measure GUS activity quantitatively, MUG assays were conducted on each agroinfiltrated leaf $(1-2 \text{ cm}^2)$ as described by Jefferson *et al.* (1987). The reaction was stopped after 5, 10, 15, 20, and 30 min. Fluorescence was measured at these different times on a FLUO-star plate reader (BMG Labtechnologies Inc.) at 460 nm when excited at 355 nm. Values from the fluorescence assay were standardized by protein concentration, determined as described by Bradford (1976).

Results

Gibberellins block floral initiation in spring in once-flowering rose

To study the effect of GA treatment on plant development, exogenous application of GA_3 at different concentrations (30, 70, and

140 $\mu M)$ was performed in spring on the OF roses, RW and FP, and its CF mutant LWP (Fig. 1).

On non-treated RW, 80% of the emerging shoots became floral, whereas for the GA₃ treatment (30 μ M and 70 μ M), almost all new emerging shoots remained vegetative (only 5.5% and 1%, respectively, were floral). At higher GA₃ concentrations, flowering was completely inhibited (Fig. 2B). GA₃ treatment also had an effect on the vegetative development of the stem. The length of internodes increased significantly: without treatment, internodes measured 3 cm, at 30 µM GA₃ they measured 4.3 cm, and they measured 4.5 cm for the higher concentrations of GA₃ (Fig. 2A). The same inhibitory effect on blooming was shown on FP (Supplementary Table S2 at JXB online). Flowering was not inhibited by GA₃ treatment in LWP, whereas internode length was reduced. The effect of the GA synthesis inhibitor PCB was tested in FP and LWP. PCB treatment significantly reduced internode length. Concerning flowering, PCB treatment has no effect on LWP, whereas at high concentrations (70 µM and 110 µM), flowering was significantly reduced in FP. Only 50% of the shoots became floral at 110 µM instead of 79% for untreated plants (Supplementary Table S2).

To evaluate the effect of GA₃ treatment on the fate of the apex, cross-sections of the apex from the vegetative to the floral stage were taken using the OF rose, RW, treated or not with 30 µM GA₂ (Fig. 3). In untreated plants after bud outgrowth (42 DAB), the vegetative meristem was narrow with leaf primordia on its flanks (Fig. 3A). The meristem then emerged and enlarged (56 DAB; Fig. 3C). This stage has been defined as the 'floral initiation' stage. The meristem then became floral and bracts were visible (63 DAB; Fig. 3E). Later, floral organs (sepals, petals, stamens, and pistils) are visible (70 DAB, Fig. 3G). Under untreated conditions, the floral initiation took place 56 DAB. In GA₃-treated plants (Fig. 3B, 3D, 3F, 3H), the meristem was narrow and dome-shaped for all stages. Leaf primordia were visible on the flank of the meristem. This structure was typical of a vegetative meristem. These observations clearly demonstrated that GA₃ blocked floral initiation and that the meristem remained vegetative.

GA_3 is necessary during a short period after bud outgrowth to inhibit flowering.

To characterize the GA response, different durations and periods of GA₃ treatment (30 μ M) were tested before blooming in spring. To modify the duration and the period of the treatment, GA₃ application was started and ended on different dates, the 'late' and 'early' treatments, respectively (Fig. 1B). The duration varied from 20 d to 70 d, and the period was from late January to the end of March.

The length of internodes significantly increased between untreated plants (3 cm) and plants treated with GA₃ (from 3.5 cm to 4.6 cm), except for the GA₃ treatment of 20 d (2.3 cm) (Fig. 2A). In general, the longer the treatment, the longer the length of the internodes.

Concerning flowering behaviour, the longer the treatment, the higher the GA inhibiting effect. For GA-treated roses, 5.5, 14.8, 25.5, and 40.5% of the shoots became floral for 70, 63, 47, and 33



Fig. 2. Effect of GA₃ treatment on average internode size in centimetres (A) and percentage of shoots that become floral (B) on $R.\times$ wichurana (RW). RW plants were treated with GA₃ (0, 30, 70, or 140 μ M) for 70 d, and with GA₃ (30 μ M) for different durations and periods. Data were the means of 10 plant observations. There was a statistically significant difference (Waerden's test, P < 0.05; and the χ^2 test, P < 0.0033, for the comparison of percentages) between means with different letters.

d, respectively, of treatment begun on different dates (Fig. 2B). For treatment terminated on different dates ('early' treatment), the same trend was observed: 80% without treatment; 16.5, 3.6, and 3% for 20, 29, and 48 d, respectively. It is interesting to note that for the same duration of treatment, differences are observed if treatments are not carried out in the same period. For 47/48 d of treatment, the percentage of floral shoots was 3% for 'early' treatment, whereas it was 25.5% for 'late' treatment. For 33/29 d of treatment, the percentage of floral shoots was 3.6% for 'early' treatment, whereas it was 40.6% for 'late' treatment (Fig. 2B). 'Early' treatments were more effective at inhibiting flowering than 'late' treatment. For example, an 'early' treatment of 20 d had the same efficiency as a 'late' treatment of 47 d (15% blooming, Fig. 2B). In the 'early' and 'late' treatments, the non-floral shoots remain vegetative and present an indeterminate growth. GA₃ blocked the floral initiation and the meristem remained vegetative. In conclusion, a 1 month treatment in February (during floral transition) is sufficient to inhibit floral transition almost completely.

Floral gene expression in response to GA₃ treatment

To examine whether the inhibition of flowering observed after GA₃ treatment could be associated with the modification of floral gene transcript accumulation, this accumulation in RW shoots that emerged in spring and after blooming using was analysed by qPCR (Figs 1, 4). In spring, in the absence of treatment, the transcripts of the floral genes, RoFT, RoFD, RoLFY, and RoSOC1, were accumulated in shoots, which then become floral. RoFT transcripts were progressively accumulated in two steps as previously described (Remay et al., 2009). The first accumulation occurred early during the floral transition (15-35 DAB; Fig. 4C), and the second occurred after floral initiation. RoFD was transiently accumulated early before floral initiation (15 DAB; Fig. 4B). The accumulation of RoSOC1 transcripts was progressive during floral transition and occurred before the late accumulation of the RoLFY and RoAP1 transcripts (70 DAB; Fig. 4). The accumulation of *RoKSN* transcripts was low in spring (Fig. 4A).



Fig. 3. Longitudinal cross-section through apices treated with GA₃ (30 μ M; B, D, F, and H) or not (A, C, E, and G). In the untreated plant, the vegetative meristem (A: 42 DAB) progressively changed into a floral meristem (C, E, and G; 56, 63, and 70 DAB, respectively), whereas in the GA₃ (30 μ M)-treated plants, the meristem remained vegetative for the same stage of development (B, D, F, and H, 42, 56, 63, and 70 DAB, respectively). VM, vegetative meristem; LP, leaf primordia; FM, floral meristem; S, sepal; FB, floral bud; P, petal; St, stamen; Pi, pistil. Scale bar=100 μ m. (This figure is available in colour at *JXB* online.)

When GA₃ was exogenously applied at 30 μ M, the expression patterns of floral genes were modified. GA₃ treatment strongly increased the accumulation of *RoKSN* transcripts. After 70 d of treatment, the *RoKSN* transcript level increased 40-fold in treated plants (Fig. 4A). *RoFT* transcript accumulation followed the same expression pattern as in untreated plants, but the accumulation was lower in treated plants than in untreated plants (Fig. 4C). The transient expression of *RoFD* (15 DAB) was not detected in treated plants. *RoSOC1* presented a transient accumulation similar to that of untreated plants, except after 70 DAB when a 2-fold transcript decrease was observed (Fig. 4E). There was no accumulation of *RoLFY* and *RoAP1* transcripts at the end of the experiment.

In FP, *RoKSN* transcripts were accumulated in response to GA₃ after a few hours. *RoLFY* transcripts were reduced 2-fold and GA₃ treatment had no effect on *RoFT* (Supplementary Table S3 at *JXB* online). To test whether floral gene transcription was *RoKSN* dependent, the expression of these floral genes was studied in the CF rose, LWP. After GA₃ treatment, the expression of *RoFT*, *RoSOC1*, and *RoAP1* was not affected, whereas expression of *RoLFY* and *RoFD* was reduced 2-fold (Fig. 4G). No *RoKSN* transcript could be detected.

GA₃ and PCB treatment do not modify the fate of indeterminate vegetative shoots after blooming.

In OF roses, new emerging shoots were vegetative after the blooming in spring (Fig. 1A), whereas in CF roses new shoots were always terminated by an inflorescence. Since GA₃ inhibits flowering in spring, it was investigated whether a GA synthesis inhibitor, PCB, could allow new blooming later in the season in RW (Fig. 1A). Treatments with PCB were performed at different concentrations (14, 56, and 112 μ M), and with GA₃ (30 μ M) (Table 1). At 30 μ M GA₃, a significant increase in internode length (2.5 cm) was observed compared with untreated plants (2 cm). In contrast to the GA₃ treatment, PCB treatment significantly reduced the internode length at the three concentrations tested in RW and LWP. In RW, the length of internodes was 2 cm for untreated plants and 1.6, 1.5, and 1.4 cm for plants treated with PCB at 14, 56, and 112 μ M, respectively. The same trend was observed in LWP (Table 1). Neither of the treatments (GA₃ or PCB) had an effect on flowering. Under all conditions, in RW, the shoots remained vegetative; no flowers were observed. In LWP, all the shoots were floral (Table 1).

In the absence of treatment, transcript accumulation of *RoFT*, *RoSOC1*, *RoFD*, *RoAP1*, and *RoLFY* remained low, and *RoKSN* transcripts were accumulated (Fig. 4). After exogenous application of GA₃, *RoKSN* expression increased 3-fold. GA₃ treatment had no effect on *RoFD* and *RoFT* (Fig 4B). *RoLFY* transcript decreased 3-fold after 34 d of GA₃ treatment (Fig. 4D), while *RoAP1* was accumulated later (34 DAB). No significant changes were detected after PCB (at 56 μ M) treatment, except for *RoFD* and *RoFT*, which were transiently induced at 14 DAB (Fig. 4B).

RoKSN promoter contains GA-responsive cis-elements

The induction of *RoKSN* by GA was further investigated by studying the upstream genomic region of *RoKSN*. A 1200 bp sequence



Fig. 4. Transcript accumulation of floral genes in spring and later in summer in GA₃-treated (squares), PCB-treated (triangles), or untreated RW (diamonds). Transcript accumulation of floral genes was followed by qPCR in axillary shoots for (A) *RoKSN*, (B) *RoFD*, (C) *RoFT*, (D) *RoLFY*, (E) *RoSOC1*, and (F) *RoAP1*. The *x*-axis indicates the number of days at which apices were harvested after the beginning of the GA₃ or PCB treatment. The transcript accumulation levels are expressed in relation to the first sample, harvested in January, for each gene according to the Pfaffl ratio (Pfaffl, 2001) (base value=1);. The grey box represents the floral initiation determined according to the histological analysis (Fig. 2). Kruskall–Wallis test on two biological replicates (P < 0.05) was realized, and asterisks represent statistical differences between GA₃-treated and untreated plants; triangles represent statistical differences between PCB-treated and untreated plants. (G) Transcript accumulation of floral genes in GA₃-treated (grey box), and untreated (black box) LWP. The transcript accumulation levels are expressed in relation to the untreated plants. Data with different letters indicate a significant difference (Kruskall–Wallis test P < 0.05). nd, not detected

Table 1. Effect of GA ₃ and the gibberellin inhibitor (PCB) on floral development and vegetative growth in late summer of OF rose)
(R.×wichurana, RW) and CF rose ('Little White Pet', LWP).	

Genotype	T	Second treatment (August-September)							
	Ireatment	Untreated	GA ₃ 30 μM	РСВ 14 µМ	РСВ 56 µМ	PCB 112 μM			
	Average internode size (cm)	2±0.5 b	2.5±0.7 ª	1.6±0.5 °	1.5±0.8°	1.4±0.4°			
	Percentage of shoots that become floral	0	0	0	0	0			
LWP	Average internode size (cm)	2.3 ± 0.7 ^b	2.6±0.5 ª	2.1 ± 0.5 ^b	$1.7 \pm 0.5^{\circ}$	1.5 ± 0.5^{d}			
	Percentage of shoots that become floral	99.2 ^a	99.1 ^a	99.1 ^a	100 ^a	100 ^a			

Data were the means of five plant observations. There was a statistically significant difference (Waerden's test; P < 0.05) between means with different letters. The experimental design is described in Fig. 1.

of *RoKSN* (upstream of the ATG codon) was isolated. By comparing promoters of *TFL1* of other *Rosaceae* (*Malus domestica*, *Prunus persica*, and *Fragaria vesca*) and *Arabidopsis thaliana*, two conserved regions were detected (Fig. 5A). Block A (-350 bp to -281 bp) was specific to the *Rosoideae* subfamily, whereas block B (-177 bp to -61 bp) was common to the *Rosaceae* family. One GAMYB *cis*-element, known to be involved in the GA response (Gocal *et al.*, 1999), was identified in block A. No GA response elements were found in block B. The non-conserved regions of the promoter contained three PIF3, three GAMYB, 10 WRKY71, one CARE, and one GARE element (Supplementary Fig. S1 at *JXB* online)

To determine the functional region for GA response expression, successive promoter deletions were performed: p200, p400, p800, and p1200, which corresponded to the -200, -400, -800, and -1200 bp of the upstream sequence of *RoKSN*, respectively. No fluorescence or GUS activity was detected with the p200 construct that contains only a TATA box element (Fig. 5C). Weak expression was detected with the p400 promoter in the absence of GA. In the presence of GA, the promoter activity was significantly increased by 14-fold (Fig. 5C). With the p800 and p1200 constructs, the promoter activity was not significantly different between GA-treated and non-treated plants. In both conditions, GUS activity was high (Fig. 5C). The 800 bp fragment may contain a positive *cis*-element, which provokes a constitutive expression of the promoter under the conditions used here (heterologous system). Due to this constitutive expression, GA induction may no longer be detectable.

Discussion

Physiological studies have demonstrated that GAs are floral repressors in woody perennials such as fruit trees (Wilkie *et al.*, 2008). However, how GA mediates its inhibitory effect at the molecular level remains largely unknown. In this study, the role of GAs in flowering on OF roses was investigated at the physiological and molecular level. It was demonstrated that the inhibitory effect of GA in rose is principally mediated by a floral repressor homologue to *TFL1*, *RoKSN*.

GA treatment modified the phenology of flowering in once-flowering rose

Under the experimental conditions without GA treatment, the OF roses (RW and FP) initiated their blooming in mid-March. The

histological analysis showed that the vegetative meristem was progressively converted into a floral meristem (Fig. 3). As previously described by Roberts et al. (1999), GA3 treatment inhibited flowering in OF roses. High levels of GA_3 (>30 μ M) completely blocked flowering (Fig. 2). The histological analysis showed that GA₃ treatment inhibited floral initiation. After GA₃ treatment, the apex remained vegetative and none of the morphological modifications observed during floral initiation (such as enlargement and doming of the meristem) was observed (Fig. 3). To specify the effect of GA₃, different times and durations of GA₃ application were used. A 1 month treatment before floral initiation (in February) was sufficient to obtain a strong inhibition of flowering (Fig. 2A). This period was referred to as floral transition, the phase when the meristem is still vegetative but receptive to signals that stimulate or inhibit flowering. During this phase, GA₃ acts as a floral repressor. This is consistent with the role of GA, which is known to promote vegetative growth instead of reproductive development in woody plants. The exogenous application of GA in fruit trees blocked the floral process and partially reduced the intensity of flowering, for example in apple (Tromp, 1982; Bertelsen et al., 2002) or in avocado (Salazar-Garcia and Lovatt, 2000). In peach, this inhibitory effect has been linked to the date of treatment (Painter and Stembridge, 1972). In strawberry, GA treatment induced the initiation of runners and repressed the formation of crown branches (Hytönen et al., 2009). None of these studies explained how GA mediates its inhibitory effect on flowering.

A floral repressor, RoKSN, is transcriptionally regulated by GAs

To look for the molecular targets of GA during the rose floral process, molecular analyses were carried out in spring and after blooming in GA₃-treated and untreated OF rose. During floral transition in untreated plants, floral activators were progressively accumulated (*RoFT* and *RoSOC1*). Later, *RoLFY* and *RoAP1* were accumulated at the end of floral initiation when the meristem started to become floral (Figs 3, 4). *RoKSN* transcripts remained low during floral transition and initiation. Similar patterns of transcript accumulation have already been observed in FP, an OF rose (Remay *et al.*, 2009; Iwata *et al.*, 2012), and in other *Rosaceae*. In apple, where floral initiation takes place in June after blooming, expression patterns of floral activators (homologues of *SOC1*, *FT*, *LFY*, and *AP1*) and repressors (homologues of *TFL1*) are similar (Hättasch *et al.*, 2008).




When GA₃ was exogenously applied, the strongest effect was the large accumulation of *RoKSN* transcripts and the absence of the accumulation of *RoLFY* and *RoAP1* transcripts (Fig. 4). In RW, *RoKSN* was accumulated 20 times more in GA₃-treated plants than in untreated plants in spring (Fig. 4). The same pattern was observed in summer. Furthermore, *RoKSN* was induced by GA₃ in two independent OF roses, RW and FP, suggesting a conservation of this regulation in rose (Supplementary Table S3 at *JXB* online).

To analyse the induction of *RoKSN* by GA₃ further, promoter analysis was performed. A 200 bp fragment (located between -200 bp and -400 bp) is sufficient to induce expression of *RoKSN* in response to GA₃. Indeed, the 200 bp promoter is not responsive to GA₃, whereas the 400 bp promoter revealed a stronger activity in the presence of GA_3 (Fig. 5C). This part of the promoter contained GAMYB and PIF3 cis-elements (Supplementary Fig. S1 at JXB online). It is hypothesized that the transcription factor PIF3 (PHYTOCHROME INTERACTING FACTOR 3) integrates different endogenous and environmental factors such as light and GA to induce stem elongation (Feng et al., 2008). In Arabidopsis, AtMYB33, a GAMYB transcription factor, is able to link to the GAMYB-binding site in the LFY promoter (Gocal et al., 2001). This 200 bp fragment corresponds to block A, conserved between rose and strawberry (Fig. 5A), suggesting that GA regulation might be conserved in strawberry where GA is also known to inhibit flowering (Thompson and Guttridge, 1959).

By studying transcript accumulation and promoter activity, the induction by GA₃ of *RoKSN*, a gene of the *TFL1* family, was clearly demonstrated. Further investigations are necessary to understand the signalling between GAs and *RoKSN*. This study represents the first demonstration of the induction of a floral repressor by GA. In *Arabidopsis*, *TFL1* belongs to a small family (Kobayashi *et al.*, 1999). It was recently demonstrated that other members of the *TFL1* family were regulated by hormones. The paralogues of *FT*, *TWIN SISTER OF FT*, were activated by cytokinin (BAP) to induce flowering under SDs (D'Aloia *et al.*, 2011). In *A. thaliana*, *MOTHER OF FT* and *TFL1* regulated seed germination via abscisic acid and the GA pathway (Xi *et al.*, 2010). During floral initiation, it was hypothesized that *FT* was induced by GA via a positive regulation of *CO* (Hisamatsu and King, 2008; Porri *et al.*, 2012).

RoKSN mediates floral inhibition by GA

GA blocked the floral transition in OF roses (Fig. 2), and *RoKSN*, a floral repressor, is induced in response to GA₃ (Fig. 4). It was uncertain whether this induction by GA might be the principal component in GA floral inhibition. During the floral process, the GA response between CF and OF roses was different: after GA₃ treatment, OF roses did not flower, while CF roses flowered (Roberts *et al.*, 1999; this study). The CF roses had no functional *RoKSN* allele, due to insertion of a retrotransposon (Iwata *et al.*, 2012). As a consequence, CF roses did not accumulate *RoKSN* and were no longer able to respond to GA. No floral repression occurred. In OF roses, during the floral transition, *RoKSN* is weakly accumulated and floral initiation can happen (Fig. 4A). The low accumulation may be explained by the low GA content (Roberts *et al.*, 1999) and the regulation of GA metabolism

genes: transient accumulation of *RoGA2OX*, a gene encoding an enzyme involved in GA inactivation, and low accumulation of *RoGA20OX*, a gene encoding an enzyme involved in the last steps of active GA synthesis (Remay *et al.*, 2009).

After exogenous application of GA₃, floral genes were downregulated (such as *RoSOC1*, *RoFD*, *RoLFY*, and *RoAP1*) (Fig. 4). To determine whether these regulations were *RoKSN* dependent, the expression of these genes in response to GA₃ was studied in a CF rose, LWP (Fig. 4G). In the absence of *RoKSN*, transcript accumulation of *RoFT*, *RoSOC1*, and *RoAP1* was not modified after GA₃ treatment. In contrast, *RoLFY* and *RoFD* transcripts were reduced 2-fold after GA₃ treatment. These results suggest that *RoFT*, *RoSOC1*, and *RoAP1* regulation by GA is *RoKSN* dependent, whereas the GA effect on *RoFD* and *RoLFY* is, at least partially, independent of *RoKSN*.

The following model is proposed to explain the floral inhibition by GA in OF roses. During the floral transition, exogenous application of GA₃ leads to a strong accumulation of RoKSN that blocks induction of floral genes such as RoAP1, RoFT, and RoSOC1 (Fig. 4). As proposed in Arabidopsis, it can be hypothesized that RoKSN is interacting with RoFD to block induction of downstream floral genes such as RoSOC1 and RoAP1 (Hanano and Goto, 2011). The RoFT repression could be due to a positive feedback loop that is blocked in the presence of RoKSN. Such a positive feedback loop has been shown for TSF, the paralogue of FT in Arabidopsis (Yamaguchi et al., 2005). RoKSN is not the only target of GA. RoFD and RoLFY are down-regulated in the absence of RoKSN, suggesting an independent pathway for these genes. However this *RoKSN*-independent pathway seems to be minor, as in roksn mutants (such as as LWP, a CF rose), no GA floral inhibition happens even at high GA₃ concentrations (Supplementary Table S2 at JXB online). The transient expression of RoFD (15 DAB, Fig. 4B) is early and may suggest a role for RoFD in processes other than floral initiation. FD is interacting with TFL1 and FT (Wigge et al., 2005; Hanano and Goto, 2011). However, a role for FD in other developmental process cannot be excluded as FD homologues can interact with proteins controlling processes such as growth (Pnueli et al., 2001; Mimida et al., 2011).

This is the first model that focuses on the inhibition of flowering by GA. Previous studies concerning the role of GA in flowering mainly targeted plants where GA had a stimulating effect. In these plants, for example *Arabidopsis* and *L. perenne*, GA acts via activation of floral activators (Blazquez *et al.*, 1998; Gocal *et al.*, 2001; Moon *et al.*, 2003; MacMillan *et al.*, 2005).

GA might not be the only factor that regulates *RoKSN*. After flowering in spring, the *RoKSN* level increases and blooming is blocked (Iwata *et al.*, 2012; this study). Treatment with PCB, a GA synthesis inhibitor, fails to induce new flowering (Table 1). Reduction of GA content is not sufficient to induce flowering. The pattern of floral gene accumulation is not modified after PCB treatment (Fig 4). It cannot be excluded that PCB treatment did not reduce GA content enough to induce blooming (as no GA dosage study has been performed). However, PCB treatment is sufficient to reduce internode length (Table 1; Supplementary Table S2 at *JXB* online). It is proposed that after the blooming in spring, *RoKSN* could be regulated by GA but also by other pathways. Vernalization is a potentially interesting pathway as OF roses maintained in a greenhouse without a cold period do not flower the next spring. Control of *TFL1* homologues by another environmental pathway has already been proposed. In diploid strawberry, the expression of *FvKSN*, the orthologue of *RoKSN*, is regulated by the photoperiodic pathway. Under SDs, expression of *FvKSN* is repressed to permit floral initiation, whereas under LDs, expression of *FvKSN* increases to inhibit flowering (Koskela *et al.*, 2012). Furthermore, in *Arabis alpina*, *AaTFL1* is involved in an 'age-dependent response to vernalization' (Wang *et al.*, 2011). In young shoots, *AaTFL1* acts as a floral repressor after vernalization and blocks *LFY* expression. In old shoots, *AaTFL1* increases the duration of vernalization required for flowering (Wang *et al.*, 2011).

In conclusion, a model has been proposed that reveals how GA can inhibit flowering in rose by positively regulating a floral repressor, *RoKSN*. Pathways other than GA such as vernalization may control blooming in rose, and further experiments are required to explore the molecular interactions between these different pathways.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Location of GA response elements in the promoter of *RoKSN*.

Table S1. List of the names and sequences of primers used in the study.

Table S2. Effect of GA_3 and a gibberellin inhibitor (PCB) on OF rose (Félicité et Perpétue; FP) and CF rose (Little White Pet; LWP) on floral development and vegetative growth in spring during floral transition

Table S3. Induction of *RoKSN*, *RoLFY*, and *RoFT* by gibberellins (GA₃) in *R. hybrida* 'Félécité et Perpétue'.

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The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry

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SUMMARY

Flowering is a key event in plant life, and is finely tuned by environmental and endogenous signals to adapt to different environments. In horticulture, continuous flowering (CF) is a popular trait introduced in a wide range of cultivated varieties. It played an essential role in the tremendous success of modern roses and woodland strawberries in gardens. CF genotypes flower during all favourable seasons, whereas once-flowering (OF) genotypes only flower in spring. Here we show that in rose and strawberry continuous flowering is controlled by orthologous genes of the *TERMINAL FLOWER 1 (TFL1)* family. In rose, six independent pairs of CF/OF mutants differ in the presence of a retrotransposon in the second intron of the *TFL1* homologue. Because of an insertion of the retrotransposon, transcription of the gene is blocked in CF roses and the absence of the floral repressor provokes continuous blooming. In OF-climbing mutants, the retrotransposon has recombined to give an allele bearing only the long terminal repeat element, thus restoring a functional allele. In OF roses, seasonal regulation of the *TFL1* homologue may explain the seasonal flowering, with low expression in spring to allow the first bloom. In woodland strawberry, *Fragaria vesca*, a 2-bp deletion in the coding region of the *TFL1* homologue introduces a frame shift and is responsible for CF behaviour. A diversity analysis has revealed that this deletion is always associated with the CF phenotype. Our results demonstrate a new role of *TFL1* in perennial plants in maintaining vegetative growth and modifying flowering seasonality.

Keywords: retrotransposon, seasonality, floral initiation, perennial plants, ornamentals.

INTRODUCTION

Obtaining plants that flower over a long period is the goal of many gardeners, so as to be able to achieve year-round fruit and flower production. The flowering period can be lengthened by exploiting the blossoming duration or number of flowering cycles. Most plants have a single annual flowering period (once-flowering habit, OF). Some perennial plants have the ability to flower again during the year: they can flower continuously during the favourable season (continuous flowering habit, CF) or they may only have a second bloom later in the season, which may be occasional (occasional re-blooming habit, OR). In some Rosoidae, such as rose and strawberry species, the CF trait is present, and is controlled by a recessive major gene named *RECURRENT BLOOMING* (*RB*) and *SEASONAL FLOWERING LOCUS* (*SFL*) in diploid rose (Semeniuk, 1971) and woodland strawberry (*Fragaria vesca*) (Brown and Wareing, 1965; Albani *et al.*, 2004), respectively. In rose and woodland strawberry, the origin of continuous flowering is uncertain. The phenotype may have arisen from a mutation in natural populations. The rose mutant was thought to be selected from wild *Rosa chinensis* in China (Hurst, 1941), used in rose breeding as early as the Song Dynasty (AD 960– 1279; Ogisu, 1996). Later, in the 18th century, cultivated CF Chinese roses were introduced to Europe and crossed with OF European roses to give rise to modern roses (Hurst, 1941). Although mentioned as early as 1553, the 'perpetual' woodland strawberry, native to the Alpine mountain region, was clearly described in 1766 and then introduced to gardens all over Europe (Duchesne, 1766). These *F. vesca semperflorens* plants were considered as mutants of OF woodland strawberry (Darrow, 1966). The potential of continuous flowering for flower and fruit production from spring to late fall explains the high gardening success of these CF roses and woodland strawberries.

In rose and woodland strawberry, the CF habit affects not only flowering but also other important developmental characters: CF plants have a short juvenile phase and flower rapidly after seed germination (Duchesne, 1766; Vries, 1976). In CF roses, all shoots are terminated in an inflorescence (determinate growth) (Figure 1b; Vries, 1976). In OF roses, the primary shoots remain vegetative with indeterminate growth, and flowering only occurs on axillary secondary shoots (Figure 1a). In woodland strawberry, the terminal meristem differentiates an inflorescence. Axillary meristems ensure the continuity of vegetative development in the OF habit, whereas in the CF habit, they also differentiate inflorescences as long as the growing conditions are favourable (Brown and Wareing, 1965; Savini et al., 2006). In OF roses and woodland strawberry, flowering is under environmental control (Battey *et al.*, 1998; Foucher *et al.*, 2008). CF roses are self-inductive and need no environmental control for flowering (Halevy, 1972). There have been conflicting reports on the environmental control of flowering in CF woodland strawberries, which could have lost photoperiodic and thermal control (Battey *et al.*, 1998), or could have become long-day plants (Sonsteby and Heide, 2008).

In Chinese rose and woodland strawberry, as the mutation leading to CF is recessive and affects flowering and determinate/indeterminate growth, good candidate genes are TERMINAL FLOWER 1 (TFL1) homologues. Indeed TFL1 homologues control shoot meristem identity by repressing the floral transition. TFL1 homologues are required to maintain the inflorescence identity of the shoot apical meristem: examples are Arabidopsis thaliana (Bradley et al., 1997; Ratcliffe et al., 1998), Antirrhinum majus (Bradley et al., 1996), Pisum sativum (pea; Foucher et al., 2003) and Glycine max (soya bean; Liu et al., 2010; Tian et al., 2010). Furthermore, in Arabidopis and pea, TFL1 also influences the length of the vegetative phase (Bradley et al., 1997; Foucher et al., 2003). In Arabidospis, tfl1 mutants flower earlier. In perennial tomato, SELF PRUNING, the homologue of TFL1, controls the length of the sympodial unit. After flowering, sp mutants produce only a few sympodial units, with fewer and fewer nodes, leading to a terminal inflorescence with no repetition of the sympodial unit (Pnueli et al., 1998). In



Figure 1. Different blooming modes in rose.

(a) once-flowering genotypes (as *Rosa wichurana*) flower once a year in spring. Terminal inflorescences are borne by lateral shoots, arising from axillary buds of shoots from the previous year. Then, after the first blooming, new developing shoots (especially basal, reiteration shoots) have an indeterminate vegetative growth. In the next spring, from these shoots, axillary shoots will develop and terminate in an inflorescence.

(b) Continuous flowering genotypes (such as the old Chinese cultivated variety 'Old Blush') flower during the growing seasons. All growing shoots terminate in an inflorescence (determinate growth).

(c) Occasionally re-blooming (OR) genotypes, such as the vegetative mutants of continuous flowering genotypes (sports) 'Pompon de Paris Climbing', flower in spring from axillary shoots that develop terminal inflorescences. After spring, the new arising shoots have an indeterminate vegetative growth. Occasionally, the new arising shoots produce axillary shoots with terminal inflorescences during the late summer and early autumn (OR habit). Double and single lines represent shoots from the previous and current years, respectively. Arrows represent indeterminate vegetative growth. Flowers are represented by circles. For the three phenotypes, the duration of flowering is presented by a blue box. Occasional blooming is represented by a blue box with dotted lines.

Populus sp. (poplar), TFL1 does not modify indeterminate growth, but rather regulates the first onset of flowering, axillary meristem identity and dormancy release (Mohamed et al., 2010). In Vitis vinifera (grapevine), a tfl1 mutant causes a modification in the inflorescence architecture (Fernandez et al., 2010). In Arabidposis thaliana, TFL1 belongs to a larger family, represented by six proteins (Kobayashi et al., 1999). Among them, FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) are floral activators (Kardailsky et al., 1999; Kobayashi et al., 1999). Until recently, the biochemical nature of florigen remained elusive, until breakthrough experiments in A. thaliana (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Notaguchi et al., 2008), Solanum lycopersicum (tomato; Lifschitz et al., 2006), Oryza sativa (rice; Tamaki et al., 2007) and Cucurbita moschata (pumpkin; Lin et al., 2007) revealed that the protein encoded by the FT gene is the florigen.

Here we investigated the function of *TFL1* homologues in Rosoidae, and we demonstrated that continuous flowering in rose and woodland strawberry is controlled by a *TFL1* homologue.

RESULTS

SEASONAL FLOWERING (SFL) and RECURRENT BLOOM-ING (RB) are orthologous loci in strawberry and rose

As rose and strawberry are phylogenetically closely related species (both belonging to the Rosoideae tribe of the Rosaceae family; Potter et al., 2007), and as the genetic control is the same in both (recessive gene in both cases), we attempted to determine by comparison mapping if the two loci controlling continuous flowering in rose and woodland strawberry, i.e. RB and SFL, respectively, are orthologues. RB and SFL were mapped on the rose linkage group RG3 and the strawberry linkage group FG6, respectively (Sargent et al., 2006; Spiller et al., 2011). Eight Rosa markers located on RG3 had one significant match to the Fragaria genome, with a BLASTN score of <E-11. These eight Fragaria matches were located on the Fragaria chromosome FG6 (Figure S1). The colinearity of these loci was conserved, except for two loci that displayed small rearrangements. Therefore, a large segment of rose linkage group RG3 was orthologous to the upper part of the strawberry FG6, which gave rise to the hypothesis of an orthologous relationship between the genes controlling recurrent flowering in Rosa and Fragaria.

RoKSN and *FvKSN* are *TFL1* homologues and co-localise with the *RB* and *SFL* loci, respectively

Using degenerate primers in rose and results from the strawberry genome sequence (Shulaev *et al.*, 2011), we isolated four and seven *TFL1/FT* homologues in rose and strawberry, respectively. We identified two *TFL1* homologues in rose and strawberry as good candidates for the continuous flowering gene, termed *KSN* (Koushin, an old

Japanese name for the Chinese CF rose cultivar). RoKSN and FvKSN clearly belonged to the TFL1 clade, along with RoT-FL1 and FvATC; whereas RoFT, FvFT, FvFT2 and FvFT3 were included in the FT clade and one (FvMFT) in the MFT clade (Figure 2). We also identified two TFL1/FT homologues (RoTFL1b and FvTFL1b) that are closely related to AtBFT.

Using large populations that segregated for the OF/CF trait, we found that *RoKSN* and *FvKSN* co-localized with the *RB* and *SFL* locus in rose and woodland strawberry, respectively. No recombinant was found between the *RoKSN/FvKSN* and *RB/SFL* loci in 670 individuals from two F₁ progenies obtained via the cross between CF and OF roses, and in 158 S₁ selfed progeny of *F. vesca* llaria, respectively.

Insertion of a retrotransposon in *RoKSN* is responsible for the recurrent blooming character

By analysing seven CF roses of independent origins, ascertained by pedigree (Cairns, 2000), we detected the presence of a retrotransposon in the second intron of *RoKSN* (Figure 3). All CF roses were homozygous for the *RoKSN* allele with the retrotransposon. The 9-kbp long retrotransposon presented characteristics of the *copia* retrotransposon family (Kumar and Bennetzen, 1999). This large insertion may lead to an inactive *RoKSN* allele (see results below).

In order to validate the function of *RoKSN* in continuous flowering control in roses, we studied vegetative mutant pairs showing different flowering behaviours. Frequently, CF roses mutate into climbing roses (Lewis, 1994). These climbing roses have primary shoots with indeterminate vegetative growth, whereas inflorescences are produced by axillary secondary shoots. They bloom in spring, and occasionally they can flower later in autumn (OR; Figure 1c). Only one mutation from OF to CF was described. An OF rose, i.e. 'Félicité et Perpétue' (FP), gave a CF dwarf mutant 'Little White Pet', LWP (Roberts et al., 1999). Each of the seven mutants studied showed a difference at the RoKSN locus when compared with the wild type. All six climbing mutants had a new allele at the RoKSN locus with a 1-kbp insertion at the same place as the 9-kpb insertion in the second intron (Figure 3). This 1-kbp sequence corresponded to the long terminal repeat (LTR) element of the retrotransposon. Five climbing mutants ('Gold Bunny Cl.', 'Wendy Cusson Cl.', 'Pink Chiffon Cl.', 'Iceberg Cl.' and 'Peace Cl.') had both the 9-kbp retrotransposon and 1-kbp LTR insertions. One climbing mutant ('Old Blush Cl.') only had the 1-kbp insertion (Figure 3). Mutation in climbing roses can be explained by the recombination of the retrotransposon in the climbing mutants. This recombination restores an active RoKSN allele (see discussion below). In the rare OF/CF mutation, FP was found to be heterozygous at the RoKSN locus (allele with and without the retrotransposon), whereas LWP only had the allele with the retrotransposon (Figure 3c). In LWP, by sequencing and Southern blot analyses, only one allele has been found, i.e. that with the retrotransposon (Figures 3c



Figure 2. Phylogenetic analysis of members of the FT/TFL1 family in rose and woodland strawberry. The TFL1 homologues RoKSN and FvKSN belong to a small gene family. RoKSN (HQ174211), FvKSN (HQ378595), RoTFL1 (FM999796) and FvATC (21992) belong to the TFL1 clade. Four homologues [FvFT1 (04680), EvET2 (28959), EvET3 (21535) and BoET (FM999826)] belong to the FT clade, one homologue [FvMFT (09405)] belongs to the MFT clade and two more homologues [FvTFL1b (13304) and RoTFL1b (HQ174212)] are related to AtBFT. The tree was constructed using the neighbour-joining method with the TFL1 family protein from rose (outlined with dotted lines), woodland strawberry (outlined with solid lines). Arabidopsis (underlined; Kardailsky et al., 1999; Kobayashi et al., 1999), tomato (Carmel-Goren et al., 2003), grapevine (Carmona et al., 2007) and poplar (Igasaki et al., 2008). Bootstrap values for 1000 re-samplings are shown on each branch. Branches with a bootstrap value >700 (of 1000) are shown with a thick line. The dashed lines delineate the different clades (TFL1, FT and MFT).

and S2). In this latter genotype, the absence of the active allele (without the retrotransposon) can be explained by the deletion of the allele or a somatic chromatid exchange. Analysis of these independent mutants demonstrated that recombination of the retrotransposon restored an OF phenotype, whereas deletion of the functional allele led to CF roses.

Full-length *RoKSN* mRNA is not accumulated in *CF* roses as a result of retrotransposon insertion

We were unable to detect RoKSN mRNA accumulation in CF roses, whereas the transcript accumulated in OF roses (Figure 4b). mRNA synthesis or accumulation might be blocked by the copia-like retrotransposon insertion. To test this hypothesis, RoKSN transcript accumulation was studied by one-step reverse transcription (RT)-PCR in an OF/climbing mutant pair, i.e. 'Peace'/'Peace Climbing'. In one-step RT-PCR, cDNA synthesis and PCR were performed at the same time using RoKSN-specific primers. As described before, 'Peace Climbing' has an allele with the LTR element at the RoKSN locus (Figure 3c). Using primer combination F9XR11 (surrounding intron 1), one-step RT-PCR products were observed in both 'Peace' and 'Peace Climbing', suggesting that nascent RNA is transcribed and the first intron is spliced out in both (Figure 4a and c). Using primer combination F9XR13 (surrounding intron 2, where the retrotransposon is inserted), one-step RT-PCR products were observed in 'Peace Climbing' and in an OF rose ('Park Yellow Tea

Scent'); no product was detected in the CF rose (Figure 4c). Then, using primer combination F9 and primers in the retrotransposon (InsR3, InsR4, InsR5 or InsR8), we observed one-step RT-PCR products in 'Peace' corresponding to PCR products that would be expected if the second intron was not spliced out (Figure 4a and d). We conclude that, as a result of the retrotransposon insertion, the second intron is not spliced out in CF roses and no full-length mRNAs are accumulated. In climbing mutants, the recombination of the retrotransposon restored splicing of the second intron and an active *RoKSN* allele, leading to indeterminate growth and non-continuous flowering.

FvKSN, the *RoKSN* orthologue in woodland strawberry controls continuous flowering

Sequencing the entire *FvKSN* revealed that CF genotypes showed a 2-bp deletion in the first exon that caused a frame shift, which led to the rapid introduction of a stop codon in the putative translated protein and to a non-functional allele named *ksn* (Figure 5d). In the 158 S₁ seedlings, all homozygous individuals with a 2-bp homozygous deletion in the recessive locus (*ksn/ksn*) had a CF habit, whereas all homozygous individuals without a 2-bp deletion (*KSN/KSN*) or heterozygous (*ksn/KSN*) had an OF habit.

In order to confirm the role of the 2-bp deletion of *FvKSN* in continuous flowering, we conducted a diversity analysis between the 2-bp deletion and flowering phenotypes using 37 accessions of *F. vesca*, including 13 CF genotypes. The



Figure 3. Insertional polymorphism at the *RoKSN* locus between once-flowering and continuous-flowering (OF and CF, respectively) mutant roses. (a) Schematic representation of the *RoKSN* genomic region for the three different alleles. In the second intron, the CF allele had a 9-kbp *copia*-like retrotransposon, the 'climbing' (CI) allele just had a long terminal repeat (LTR) element, whereas the OF allele had no insertion. Exons are represented by grey boxes, introns are represented by black lines and primers are indicated with arrows.

(b) PCR amplification of *RoKSN* in Chinese roses. The CF Chinese rose 'Old Blush' (OB), a progenitor used to introduce continuous flowering, had a *copia*-like retrotransposon inserted in the second intron, whereas OF wild Chinese rose (*Rosa chinensis spontanea*, Rcs) had no retrotransposon. The climbing vegetative mutant of 'Old Blush' (OB Cl) only had the LTR element. The F1 \times R2 primer pair amplified the complete coding sequence (intron and exon) of *RoKSN*: 10 kb for the allele with the retrotransposon (*copia*), 2 kb for the allele with an LTR element and 1 kb for the ancestral allele.

(c) Characterization of the retrotransposon insertion in different vegetative mutant pairs using different primer combinations. All tested climbing mutants showed recombination of the retrotransposon with the LTR motif remaining. InsF8 × R6 only amplified the sequence with the retrotransposon (1 kbp amplification, *copia*), whereas IF3 × R9 only amplified the sequence with the LTR motif (1.4-kbp amplification, LTR) or the allele without insertion (400-bp amplification). In the CF/CI mutant pairs, the CF rose varieties and their corresponding climbing (CI) mutants are 'Gold Bunny' (GB), 'Wendy Cusson' (WC), 'Pink Chiffon' (PC), 'Old Blush' (OB), 'Iceberg' (Ic) and 'Peace' (Pe). The OF/CF mutant pair is 'Félicité et Perpétue' (FP) and its mutant 'Little White Pet' (LWP). MM, molecular marker; NC, negative control (water).

37 accessions were available from different genetic resources (Table S1). The sequencing of the entire FvKSN gene in all accessions revealed a total of six SNPs located in introns and the 2-bp deletion in the first exon, which led to six haplotypes (Table S1). Within this genetic polymorphism, only the 2-bp deletion was associated with the CF habit (Figure 5b and c). Using 11 neutral microsatellites, we investigated the population structure in these 37 accessions using STRUCTURE 2.2. The most likely number of potential clusters (K) was estimated as two. All genotypes except one were clearly ranked within one of the two groups. The first group included 17 genotypes from Europe, mainly from the Alps, whereas the second group included 19 genotypes that originated from Europe/North America (Figure 5a). All CF strawberries, with the 2-bp deletion, were included in the former group, suggesting that in F. vesca continuous flowering may have originated from Alpine genotypes.

The continuous flowering gene shows seasonal regulation in rose

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We monitored *RoKSN* transcript accumulation during the floral process by quantitative (q)RT-PCR. In OF *Rosa*

wichurana, new shoots arose from axillary buds of shoots from the previous year (Figure 1a). After a short vegetative phase (first three stages, W1, W2 and W3), these new axillary vegetative shoots underwent floral differentiation, with transformation of the apical meristem into a dome-like structure (stages W4 and W5). Then the new shoot was terminated by an inflorescence (stages W6-W9). RoKSN transcripts were barely detectable in axillary vegetative shoots before floral differentiation (stages W1-W3). RoKSN transcripts were transiently accumulated during floral differentiation (stage W4) and later during inflorescence development (stages W7 and W8; Figure 6a). During the floral process, RoFT, a floral activator, and later RoLFY, a homologue of the floral identity gene LEAFY (Remay et al., 2009), were progressively accumulated (Figure 6b and 6c). After the first flowering, new shoots arose from the base of the bush and had indeterminate vegetative growth during the rest of the growing season (Figure 1a). An 80-fold increase in RoKSN transcript accumulation was detected when spring (stage W1) and autumn (stage W10) vegetative shoots were compared (Figure 6a). After flowering, *RoFT* and *RoLFY* had the same level compared with spring vegetative shoots (stage W1; Figure 6). Therefore, low

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Figure 4. Effect of the insertion of the *copia*-like retrotransposon on splicing of the second intron of RoKSN. (a) Schematic representation of RoKSN mRNA and genomic (gDNA) region with exons (boxes numbered from 1 to 4), introns (lines) and retrotransposon (insert). Primers used are indicated by arrows. (b) Two-step RT-PCR using an F100 \times R6 primer combination (c). One-step RT-PCR using different *RoKSN* primer pairs. The F9 × R11 primer pair amplified the 100-bp product only if the first intron was spliced out, as the R11 primer spanned the first intron; the $F9\times R13$ primer pair amplified the 200-bp product only if the second intron was spliced out, as the R13 primer spanned the second intron. (d) One-step RT-PCR using primer pairs of F11 in combination with InsR8, InsR5, InsR4 and InsR3, respectively, located within the retrotransposon, F11 spanned the first intron, RT-PCR was performed on total RNA from terminal shoots of 'Peace' (P), a continuous-flowering (CF) rose, its climbing mutant ('Peace Climbing', PCI) and a once-flowering (OF) Chinese rose 'Park Yellow Tea Scented China' (PY). MM, molecular marker; NC, negative control (water).

RoKSN expression in spring was associated with floral transition and determinate growth, whereas high expression of *RoKSN* in autumn was associated with vegetative and indeterminate growth. In CF roses, functional *RoKSN* transcripts were not detected (because of retrotransposon insertion), and all new shoots terminated in an inflorescence.

DISCUSSION

A floral repressor regulates continuous flowering in rose and woodland strawberry

In rose, we demonstrated that the continuous flowering phenotype was explained by the insertion of a *copia*-like retrotransposon in the second intron of *RoKSN*, i.e. a *TERMINAL FLOWER 1* homologue. Insertion of the retro-

transposon modified RNA maturation. The second intron was not spliced out, and full-length RoKSN RNA was not accumulated (Figure 4). In six independent vegetative mutants, climbing mutant roses differed from CF roses in the size of the insertion, probably as a result of recombination between LTR elements (Figure 3). After retrotransposon recombination, only the solo LTR element remained and the second intron was spliced out, thus restoring a functional RoKSN allele (Figure 4). Such a process has been described in grapevine, where the recombination of a Ty3gypsy-type retrotransposon restores MYB transcription factors responsible for fruit colour (Kobayashi et al., 2004). In the CF/climbing mutant pair, the restoration of RoKSN in climbing mutants may not be complete as flowering can occur occasionally in the autumn (Figure 1c). The presence of the LTR element in the second intron may modify the level or the spatiotemporal pattern of RoKSN expression. Indeed, LTR elements contain signals for transcription regulation (Kumar and Bennetzen, 1999), which may modify RoKSN expression in climbing mutants. In another vegetative mutant pair (changing from OF to CF behaviour), the functional allele was deleted and only the non-functional allele was present, leading to the continuous flowering phenotype (Figure 3c and S2). Furthermore, the role of KSN in continuous flowering control was strengthened by the absence of recombination between RoKSN and the continuous flowering locus in large F₁ progenies segregating for the CF/OF phenotype (in 670 individuals). These results strongly suggest that in rose the continuous flowering gene is encoded by *RoKSN*, i.e. a *TFL1* homologue. Functional validation still needs to be performed in rose by knocking out the gene in OF roses. Presently, no protocols for the genetic transformation of OF roses are available (Debener and Hibrand-Saint Oyant, 2009). However, the conclusion drawn in rose was reinforced by the results obtained in woodland strawberry.

In F. vesca, which also showed continuous flowering in certain genotypes, we identified FvKSN, a gene showing high similarity with RoKSN (Figure 2). Using comparative mapping, we showed that the two genes were located in a syntenic and co-linear region, suggesting that they are orthologues. Furthermore, all CF individuals from an S1 segregating population had a 2-bp deletion in the coding sequence of the gene, whereas the deletion was absent in all OF homozygous genotypes. This deletion induced a frame shift and a stop codon leading to a truncated protein (Figure 5d). We propose that this 2-bp deletion in the coding sequence is responsible for continuous flowering in F. vesca. As no mutant was available in strawberry, we performed a diversity analysis. Among the 37 accessions studied, the 2-bp deletion and the continuous flowering phenotype were always associated (Figure 5). We concluded that FvKSN was the orthologue of RoKSN, and that both genes control the continuous flowering trait.

Figure 5. (a) Relationship between the genetic structure of 37 woodland strawberry genotypes, *FvKSN* genotyping and continuous-flowering (CF)/once-flowering (OF) phenotyping. Estimated genetic structure of woodland strawberry genotypes, based on 11 neutral microsatellites (Table S3). The structure is represented by vertical lines, in which the ancestry proportions of each genotype are indicated by the length of the white and grey segments.

(b) Genotypes are distributed in K = 2 ancestry groups (black and white). KSN and ksn are, respectively, the allele without and with the 2-bp deletion. The ksn/ksn or KSN/KSN genotyping of each genotype is indicated by vertical bars.
(c) CF/OF phenotyping, indicated by stars and triangles, respectively, for each genotype.

(d) Alignment of the protein encoded by FvKSN and the version encoded by the allele with the 2-bp deletion (FvKSNd2bp).



KSN and the seasonality of flowering

The main consequence of the continuous flowering mutation is the modification of flowering seasonality. Perennial plants repeatedly cycle between vegetative and floral development. Most perennial plants have a single reproductive phase that is synchronized to the changing seasons. OF plants bloom in spring or early summer, and further developments are vegetative. This seasonality might be explained by seasonal regulation of KSN. We showed a seasonal regulation of RoKSN transcript accumulation in OF roses: before the first bloom, RoKSN was barely expressed; in parallel, RoFT and RoLFY, homologues of the floral integrator FT and the floral identity gene LFY (Remay et al., 2009), were progressively accumulated (Figure 6). The low RoKSN level in spring may increase the flowering competence of the apical meristem. Opposite results have been found in other Rosaceae (Japanese pear and quince) where TFL1 homologues are expressed before floral differentiation and are repressed during floral differentiation (Esumi et al., 2007). In all cases, flowering is associated with LFY induction (Figure 6c; Esumi et al., 2007). TFL1 might be differently regulated between rose and pear. The RoKSN expression during floral differentiation (stages W4, W7 and W8; Figure 6a) suggests a role of RoKSN in inflorescence development. Genetic analysis reveals that a quantitative trait locus (QTL) involved in the inflorescence architecture is centred on the RB locus in rose (Kawamura et al., 2011).

After the first blooming, newly arising shoots remained vegetative and *RoKSN* transcripts accumulated. No *RoFT* and *RoLFY* transcripts accumulated. In continuous flowering roses, this regulation was disrupted by the absence of *RoKSN* transcript accumulation. The floral repressor was

absent and flowering occurred during all seasons. This report and a previous study in *Arabis alpina* (Wang *et al.*, 2009) highlighted the importance of floral repressors in the control of flowering seasonality. In *A. alpina*, *PERPETUAL FLOWERING 1 (PEP1)*, a homologue of the floral repressor *FLOWERING LOCUS C (FLC)*, limited the duration of flowering and facilitated the return to vegetative development. *PEP1* is transiently repressed by low temperature, thereby allowing seasonal flowering of *A. alpina* (Wang *et al.*, 2009).

KSN and other aspects of plant development

The continuous flowering mutation affects different developmental processes In rose and woodland strawberry, mutation of TFL1 homologues reduces the vegetative phase. CF genotypes can flower rapidly after seed germination (in a few weeks), whereas OF genotypes have a juvenile period of 1 or 2 years (Duchesne, 1766; Vries, 1976). Expression of TFL1 may avoid precocious flowering, as recently demonstrated in A. alpina (Wang et al., 2011). Furthermore, the extinction of the *TFL1* homologue in trees (apple and poplar) reduced the juvenile phase (Kotoda et al., 2006; Mohamed et al., 2010). In Citrus and Lolium perenne, accumulation of TFL1 transcripts in juvenile stems was proposed to prevent precocious flowering (Jensen et al., 2001; Pillitteri et al., 2004). All of these results suggest that TFL1 homologues have an important role in controlling juvenility in plants.

Continuous flowering also involves GA signalling, as CF roses are insensitive to GA for flowering, whereas GA inhibits flowering in OF roses (Roberts *et al.*, 1999). *KSN* might be an integrator of endogenous signals, such as plant hormones, although the interaction with hormones is so far unknown in *TFL1* homologues. In tomato, it was proposed

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Figure 6. Seasonal accumulation of (a) RoKSN, (b) RoFT and (c) RoLFY transcripts in the once-flowering (OF) genotype Rosa wichurana. Transcript accumulation was monitored by two-step RT-PCR in shoots emerging in spring and later in autumn. In spring, the axillary buds borne on shoots from the previous year burst open and gave new shoots. After a vegetative phase (stages W1, W2 and W3), the meristem became competent for flowering. Then floral differentiation took place (transformation of the apical meristem into a dome-like structure, stages W4 and W5), followed by the development of a terminal inflorescence (stages W6-W9). After the first flowering, new shoots arose and remained vegetative (OF genotypes; Figure 1a, stage W10). The x-axis indicates the dates at which shoot terminal parts were sampled in 2007 and the different stages (W1-W10). The transcript accumulation levels are expressed relative to the first sample for each gene (stage W1, base value = 1). Floral differentiation and inflorescence development stages were determined by histological and microscopic analysis, as previously described (Foucher et al., 2008). For the different stages, the terminal part of the shoot was harvested after rapid dissection (removal of young leaves). Tissues studied represent the shoot apical meristem, the shoot and the new developing leaves (primordia and very young leaves).

that SFT (the FT homologue) may interact with auxin to regulate the sympodial cycle and leaf architecture (Shalit *et al.*, 2009). In Arabidopsis, MOTHER OF FT AND TFL1

(*MFT*) was shown to regulate seed germination via the GA and ABA signalling pathway (Xi *et al.*, 2010).

Origin of continuous flowering in horticulture

Our results highlighted the origin of CF in two important horticultural plants. In woodland strawberry, CF was well described from the 18th century, and was proposed to have originated in the Alps (Duchesne, 1766). The hypothesis of only one mutation is strengthened by the presence of only one haplotype in CF genotypes with the 2-bp deletion in the FvKSN coding sequence, even though the OF displayed five haplotypes (Figure 5; Table S1). Only one mutation might be responsible for the CF habit in the wood strawberry genotypes studied, despite the fact it has been found growing wild at various sites in the European Alps (De Vilmorin, 1898). This hypothesis has to be validated on a larger number of genotypes. In rose, CF flowering was thought to arise from a mutation in wild Chinese rose and then introduced in old Chinese cultivated roses (Hurst, 1941; Ogisu, 1996). We hypothesize that the mutation may result from the insertion of a retrotransposon into the RoKSN locus in wild R. chinensis spontanea, as the retrotransposon was absent in the RoKSN locus in this wild species (Figure 3b), i.e. an OF rose that is supposed to be an ancestor of the original CF roses (Martyn, 2005).

In conclusion, *KSN* may be an integrator of endogenous and environmental factors. Understanding the regulation of *KSN* by these different factors may provide new prospects for controlling flower and fruit production in perennial plants.

EXPERIMENTAL PROCEDURES

Plant material

Rosa wichurana originated from the 'Jardin de Bagatelle' (Paris, France). Rosa hybrida 'Félicité et Perpétue' and its mutant 'Little White Pet' were obtained from Loubert Nursery (Les Rosiers sur Loire, France). Rosa chinensis spontanea was a gift from Mr Ogisu. 'Gold Bunny', 'Wendy Cussons', 'Pink Chiffon', 'Old Blush', 'Iceberg', 'Peace' and the corresponding climbing mutants were obtained from Hiroshima Botanical Garden (Japan), and are of independent origins (Cairns, 2000). The mutants are vegetative mutants, also known as sports. A sport of a clonally propagated plant arises as a mutant cell in the shoot meristem (Lewis, 1994). Rose F₁ progenies were obtained from a cross between R. whichurana and H190 (575 individuals; Hibrand-Saint Oyant et al., 2008) and R. wichurana and R. hybrida 'The Fairy' (95 individuals; Kawamura et al. 2011). In strawberry, the 158 S1 progeny was obtained from F. vesca llaria, issued from a cross between Fragolina di Ribera and Alpine (Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Forli, Italy). The collection of the 37 genotypes listed in Table S1 was obtained from Dresden, INRA and USDA-Corvallis.

Cloning and phylogenetic analysis

TFL1 homologues in rose were isolated by PCR on *R. chinensis spontanea* using the primers UtflF1 and UtflR1. All primers are listed in Table S2. In strawberry, *FvKSN* was first isolated using primers from rose, KSNqPCRF2 and KSNqPCRR2. Other *TFL1* homologues

were isolated using sequences obtained via *Fragaria* genome sequencing (Shulaev *et al.*, 2011). Amino acid sequences of FT and TFL1 homologues in Arabidopsis, tomato, poplar and grapevine were aligned with predicted FT/TFL1 proteins in strawberry and rose using CLUSTALW (Thompson *et al.*, 1997). A phylogenetic tree was produced from 1000 bootstrap replicates by applying the neighbour joining (NJ) method with PHILLIPS software. The phylogenetic tree was displayed using TREEVIEW (Page, 1996).

Phenotyping, mapping and genetic structure

Segregating populations were phenotyped for CF. In rose, genotypes were considered as CF when plants flowered in the first year after germination, and were confirmed by the presence of flowers in October and November over a 4-year period (2006-2009). In strawberry, genotypes were considered as CF when plants had inflorescences in late summer and fall. In the seven pairs of mutants, the climbing rose genotypes, classified as OF, may occasionally flower late in the summer. In rose, based on polymorphism caused by the retrotransposon insertion, the RoKSN genetic marker was developed using a multiplex kit (Qiagen, http://www.giagen.com) with F3, InsR3 and R1 primers. In strawberry, FvKSN was genotyped based on the 2-bp deletion identified in the coding region. The relatedness of 37 wood strawberry genotypes was studied with 11 neutral microsatellite loci (Table S3), distributed throughout the genome, using STRUCTURE software (Pritchard et al., 2000). Comparative mapping between RG III and FG VI was based on the anchorage of eight rose sequence markers, genes or microsatellites, on the strawberry genome (http://www.strawberrygenome.org) using BLASTN. Gene markers were localized precisely, whereas SSR markers were attributed to scaffolds.

Gene expression analysis

Total RNA extraction was performed as previously described by Remay et al. (2009). To test the effect of the retrotransposon on mRNA accumulation, a one-step RT-PCR was performed using the one-step RT-PCR kit (Qiagen), according to the manufacturer's recommendations. Briefly, both cDNA synthesis and PCR were performed in a single tube using gene-specific primers and target RNA. To study RoKSN accumulation during the floral process, a two-step RT-PCR was carried out as previously described (Remay et al., 2009). Total RNA was reverse transcribed using oligo-dT primers and subjected to quantitative PCR to analyse transcript accumulation. The level of RNA was normalized using the TCTP gene as a reference, after checking the homogeneity of the cycle threshold (C_t) variation with a second housekeeping gene: i.e. EF1a (Remay et al., 2009). Terminal parts of the shoots were harvested in spring and autumn from R. wichurana at different time points (see details in the legend of Figure 6). The terminal part of the shoot was dissected (removal of young leaves), and therefore corresponds to the shoot apical meristem, the leaf primordia and the stem.

ACCESSION NUMBERS

RoKSN (HQ174211); FvKSN (HQ378595); RoTFL1b (HQ174212).

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SUPPORTING INFORMATION

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

Figure S1. Comparative mapping between regions surrounding continuous flowering loci in rose and woodland strawberry.

Figure S2. Molecular characterization of two continuous-flowering (CF) and once-flowering (OF) mutants in rose: 'Félicité et Perpétue' (FP), an OF genotype, and its CF mutant 'Little White Pet'; 'Old Blush' (OB), a CF genotype and its climbing (CI) mutant, 'Old Blush Climbing' (OB CI).

 Table S1. Origin and characterization of the different woodland strawberry genotypes used in the diversity analysis.

Table S2. List of primers used for the cloning and characterization of

 KSN in rose and woodland strawberry.

Table S3. List of microsatellites (SSRs) used for the genetic diversity analysis in the diploid *Fragaria vesca*.

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Genomic Approach to Study Floral Development Genes in *Rosa sp*.

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Abstract

Cultivated for centuries, the varieties of rose have been selected based on a number of flower traits. Understanding the genetic and molecular basis that contributes to these traits will impact on future improvements for this economically important ornamental plant. In this study, we used scanning electron microscopy and sections of meristems and flowers to establish a precise morphological calendar from early rose flower development stages to senescing flowers. Global gene expression was investigated from floral meristem initiation up to flower senescence in three rose genotypes exhibiting contrasted floral traits including continuous versus once flowering and simple versus double flower architecture, using a newly developed Affymetrix microarray (Rosa1_Affyarray) tool containing sequences representing 4765 unigenes expressed during flower development. Data analyses permitted the identification of genes associated with floral transition, floral organs initiation up to flower senescence. Quantitative real time PCR analyses validated the mRNA accumulation changes observed in microarray hybridizations for a selection of 24 genes expressed at either high or low levels. Our data describe the early flower development stages in *Rosa sp*, the production of a rose microarray and demonstrate its usefulness and reliability to study gene expression during extensive development phases, from the vegetative meristem to the senescent flower.

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Introduction

Roses are widely used as garden ornamental plants and cut flowers. A few flowering traits of roses are essential for the plants commercial value. Examples of these traits are plant architecture, continuous flowering, flower development, function and senescence, scent biosynthesis, reproduction and resistance to biotic and abiotic stresses. However, little is known about the molecular mechanisms that control these traits. This dearth of information limits the scope of rational selection to improve the ornamental plants. During the past decade, using model species such as *Arabidopsis thaliana*, tobacco, *Brachypodium distachyon*, rice or maize, researchers significantly enhanced our understanding of the various aspects of plant development and resistance to biotic and abiotic stresses, and of the molecular and genetic pathways associated with these aspects. However, these model species are not suitable for the studies of other flowering traits such as recurrent blooming, scent production and double flower character. Rose represents an interesting ornamental model species to address some of these aspects.

Cultivated roses have a very ancient history. The two major areas of rose domestication were China and the peri-mediterranean area encompassing part of Europe and Middle East, where *Rosa chinensis* Jacq. and *R. gallica* L. (respectively) were bred and contributed predominantly to the subsequent selection process. Artificial crossing between Asian and European roses gave birth to "modern rose cultivars". Although testimonies and historical records have documented major crosses that led to modern roses, the genetic basis on which the modern rose cultivars are established is still poorly understood [1]. It has been reported that about 8 to 20 species out of about 200 wild species have contributed to the origin of present cultivars [2,3,4].

In *Rosa sp.*, EST sequencing has identified novel genes whose expression is associated with several rose traits [5,6] such as the

scent associated genes O-methyltransferases and alcohol acetyltransferase and floral associated genes [6,7,8,9,10,11,12,13]. EST sequences were also used to generate a rose DNA microarray comprising 350 selected ESTs [14]. Using this microarray, researchers discovered several novel floral initiation genes and flower scent-related candidate genes (i.e. germacrene D-synthase encoding genes) [15]. However, this array contains only a limited number of sequences that represent genes expressed at late petal development stages.

With publicly available rose gene sequences, we generated a microarray and studied the gene expression throughout floral development, from the initial floral transition to floral senescence. We created an annotated flower EST database corresponding to 4834 genes and used the sequences to develop an Affymetrix microarray. With this microarray, we compared the transcriptome at different floral development stages. We found a good correlation between the microarray data and real time quantitative RT-PCR (qPCR) data for selected genes whose expression coincides with early, mid and late flower development stages. This dataset can help identify new rose genes associated with floral initiation, flower development and senescence.

Results and Discussion

Staging the floral transition and flower development in *Rosa sp*

Understanding the genetic basis of flower formation in ornamental plants such as roses is particularly important for future cultivar improvement. We first analyzed the visible morphological modifications during the floral process, from the vegetative meristem to the senescent flower using three rose cultivars, Rosa wichurana, R. chinensis cv. Old Blush and R. x hybrida cv. Félicité et Perpétue. Rosa wichurana and R. chinensis cv. Old Blush, two diploid roses, are among the few roses genotypes that were used in the numerous crossings and hybridizations to create the modern roses [2,16]. For example *R. chinensis* cv. Old Blush contributed major traits, like recurrent flowering and components of the characteristic 'tea scent' of modern roses [5,9,17], and R. wichurana is a non recurrent flowering rose that contributed the climbing trait for some garden roses [17]. The third rose, R. x hybrida cv. Félicité et Perpétue (FP) is a cultivated hybrid. These three cultivars were chosen because they have very different flowering habits. For example R. chinensis cv. Old Blush was chosen to study floral organogenesis, maturation and senescence, as it flowers all year long in our greenhouse at ENS, Lyon. However, continuing flowering limits our ability to sample enough vegetative meristems for transcriptome analyses. Therefore, to collect sufficient number of meristems, we also chose non recurrent flowering roses, R. wichurana and R. x hybrida cv. Félicité et Perpétue in greenhouse and field conditions at INRA, Angers.

Rose flowers are composed of four organ types arranged in whorls, from the outer to the inner sepals, petals, stamens and carpels. Flower development stages have been determined for model plants such as *A. thaliana* [18]. However, these development stages cannot be directly applied to the rose flower development. In contrast to *A. thaliana* flowers that are composed of four concentric whorls, rose flowers are composed of one whorl of 5 sepals and multiple whorls of petals, of stamens and of carpels. Furthermore, the floral architecture of modern roses differs from that of wild-type roses. For instance, modern rose varieties exhibit double flower character of high number of petals and modified numbers of stamens and carpels, whereas wild-type roses have 5 petals. Scanning electron microscopy (SEM) was used to image floral initiation in *Rosa sp* (Figure 1). Based on these imaging data,

we divided the floral initiation process into three stages. After bud outgrowth, the vegetative meristem is dome-shaped and narrow with leaf primordia on its flanks (Stage VM1 for vegetative meristem; Figure 1A, a, d). This structure is typical of a vegetative meristem as previously described [19]. Rapidly, when the new stems have acquired three fully expanded leaves, the meristem enlarges, emerges and leaf primordia are now invisible (Stage VM2, Figure 1A, b, e). We defined this VM2 stage as "pre-floral stage". Then, the meristem becomes floral characterized by a flat, large and doming structure (Stage FM for floral meristem; Figure 1A, c, f). These morphological changes were similar in the non-recurrent flowering roses, *R. wichurana* and *R. x hybrida* cv. Félicité et Perpétue. Similar enlargement and doming of the meristem were observed during the floral initiation in other related Rosaceae [20].

Sections of floral meristem and young flower buds (Figure 1A, g-k were used to define the floral organogenesis steps in R. chinensis cv. Old Blush. Five morphologically distinct developmental stages were easily distinguished under a dissecting microscope. At flower development stage 1, the floral bud is surrounded by bracts, the floral meristem is flat and five sepal primordia are visible. Floral organs subsequently form following a radial gradient so that the most external organs are the more differentiated. At stage 2, petal primordia are apparent on the flank of the hypanthium. At development stage 3 stamens primordia appear on the flank of the hypanthium while petal primordia continue developing. At stage 4, carpel primordia are the last organs that appear in the center of the hypanthium, while the other organs continue developing. At stage 5, all floral organs are apparent, and the hypanthium starts to sink below the perianth and stamens. During the onward development stages the hypanthium continues to form and the flower becomes clearly visible (Figure 1 B). The four types of floral organs continue developing and flowers start opening (VP stage for visible petals) (Figure 1 B). Then the flower fully opens (OF stage for open flower), and finally senesces (SF stage for senescing flowers).

Rose EST database creation and Rosa1_Affymetrix custom array design

We collected the available rose genes sequences (ESTs and mRNA) and built a comprehensive database. Using sequence clustering, we generated a dataset comprising 4765 unique sequences (clusters and singletons) and deposited them in http://urgi.versailles.inra.fr/GnpSeq.

For most of the clusters, one representative EST was chosen based the following criteria. Its sequence is larger than 600 nucleotides and preferably corresponding to the 5' end gene sequence. Because the rose is highly heterozygous, such strategy should prevent using chimerical sequences that might have been obtained during the clustering process. However, 343 clusters did not meet the criteria above. For these 343 clusters, two or more ESTs representing the unique sequence were used. In total, 5175 unique rose EST sequences representing 4765 unique sequences were used for the Rosa1_Affymetrix array design and a total of 6,289 probe sets including Affymetrix control probesets were designed. The arrays were manufactured by Affymetrix (http:// www.affymetrix.com).

Array sequences annotation

We used the Blastx algorithm against the nr database to identify the best protein hits for the 5175 unique rose sequences, and analyzed these results using Blast2go software [21]. 3959 sequences (76.5%) produced a significant match with one or more entry in the database. Among the 3959 sequences, 222 (5.6%)



Visible flower (*R. chinensis*)

Figure 1. Rose flower development stages. A. (a) to (f): Morphology of the floral transition in one-time flowering roses (*R. wichurana*) Schematic representation of the different stages observed during the floral transition in spring is shown in the upper panel from a vegetative meristem (VM) to a floral meristem (FM). a to c: Light microscopy of cross section of meristems. d to f: Environmental scanning electron microscopy images. Black bar: 10 µm. (g) to (k): Rose flower organogenesis stages. Cross sections of floral meristem and young flower buds. Images representing initiation of sepals (stages 1, g), petals (stage 2, i), stamens (stages 3, h) and carpels (stage 4, j). k: hypanthium starts introverting below the floral organs (stages 5). Black bar: 50 µm (g,h,i); 200 µm (j,k). **B.** Visible rose flower stages. Pictures of rose flowers at flower bud with visible petals (stage VP), open flower stage (OF) and senescing Flower stage (SF). doi:10.1371/journal.pone.0028455.g001

could not be mapped with GO terms and 3737 had at least 1 GO term. For 1439 sequences, full automatic annotations were obtained. Analysis of GO biological process mapping showed that out of these 1439 sequences, 700 (48.6% of mapped sequences) were annotated as involved in primary metabolism processes and only 43 were annotated as putative secondary metabolism genes. 120 sequences (8.33% of mapped sequences) were mapped with the GO:0010468 annotation corresponding to regulation of gene expression. GO molecular function analysis showed that 38 sequences (2.6% of mapped sequences) had putative transcription factor activity (GO:00037000). The complete list of these sequences represented in the array, giving the first Blastx hit, the Blast2go computed annotation and gene ontology, is shown in Table S1. About 23.5% of the rose sequences produced no significant Blast hit in the gene databases. It is likely that the sequences of these genes have diverged far enough to render the annotation difficult. These highly divergent genes may have evolved functions that are be specific to the Rosa genus or Rosaceae family and are therefore of particular interest.

Gene expression associated with rose floral initiation

We analyzed the transcriptomes of *R. wichurana (Rw)* and *R. x* hybrida cv. Félicité et Perpétue (FP) during floral initiation. Specifically, we compared vegetative (VM1) to pre-floral (VM2) stages and pre-floral to floral (FM) stages (Figure 2A). Such comparisons can uncover on genes potentially involved in the control of floral initiation. The rationale is that the genes upregulated between vegetative and pre-floral buds are expected to be putative floral activators. Conversely, genes repressed between vegetative and pre-floral stages are expected to be putative floral inhibitors.

824 genes in R. wichurana and 652 genes in R. x hybrida cv. Félicité et Perpétue had a dynamic expression pattern between vegetative meristem (VM1) and pre-floral meristem (VM2) (Tables S2 and S3). Between VM1, VM2 and floral meristem (FM) stages, 302 (Rw) and 104 (FP) of these genes continued to be differentially expressed. During the VM1 to VM2 transition, 336 (Rw) and 301 (FP) genes were up-regulated between vegetative and floral stages, hence they represent candidates associated with floral initiation. 488 (Rw) and 351 (FP) genes were down-regulated and they are thus potential floral initiation repressors (Tables S2 and S3). To increase the confidence in the discovery of genes associated with floral induction, the overlapping genes from both datasets (Rw and FP) were selected. 258 differentially expressed genes during the VM1 to VM2 transition were common between FP and Rw samples. Among these genes, 222 out of 258 (86%) presented the following expression pattern. 131 genes are down-regulated between VM1 and VM2 stages and are thus putative floral repressors (top list in Table 1 and complete list in Table S4A). 91 gene are up-regulated between VM1 and VM2 stages and are thus putative floral activators (top list in Table 1 and complete list in Table S4B). Altogether, these genes are interesting candidates for studying floral initiation in Rosa sp.

Among the putative rose floral activators, the expression of the putative rose homologues of *SOC1 (RhSOC1) and APETALA1 (RhAP1)* were induced during the floral initiation both in *R. wichurana* and in *R. x hybrida* cv. Félicité et Perpétue (Tables S2 and S3; Figure 3), in agreement with previously reported data [13]. Therefore, like in *Arabidopsis* [22,23], in *Rosa sp* the expression of *RhSOC1* and *RhAP1* suggests that these genes may have similar function as floral integrator and floral meristem identity regulator, respectively. Among the genes that were differentially expressed in



Figure 2. Description of the comparisons performed using micrarrays. A. To identify genes associated with floral initiation in *Rosa* using *R. wichurana (Rw), R. x hybrida* cv. Félicité et Perpétue (FP); Comparisons were done in the 2 genotypes; VM1: vegetative meristem stage; VM2: pre-floral meristems; MF: floral meristem. **B**. Schematic representation showing the rose flower development stages from flower organogenesis (stage 1) to onset of senescing flowers (stage SF). Arrows indicates the different transcriptome comparisons. VP: flower bud with visible petals; OF: open flower; SF: Senescing flower. doi:10.1371/journal.pone.0028455.g002

both roses during floral initiation, six (BI978989, BI978732, BI978794, EC589388, BQ104046, EC586448) showed similarities to genes involved in auxin transport or auxin signalling. Two auxin-repressed homologues (BI978989 and BI978794) were down-regulated and two auxin-induced homologues (BI978732 and BI978794) were up-regulated during the floral initiation process in *Rw* and FP, suggesting dynamic auxin signalling in the rose apex during the floral initiation and the organogenesis of the inflorescence meristems. Auxin and ethylene often interact synergistically [24-25]. We found genes involved in ethylene signalling were down-regulated during floral initiation in Rw and FP. These genes (EC586386 and AY919867) showed similarities with EIN and EIL genes [26]. EIN and EIL transcription factors are positive regulators of the ethylene signalling [27]. In Arabidopsis, ethylene delayed flowering as acs mutant flowered later [28]. In addition, during the floral initiation in Rw, two genes showing similarity with ethylene synthesis gene, ACC oxydase (AF441282) and ACC synthase (BQ105189) are down-regulated. Therefore, during the floral initiation, decrease in ethylene production may lead to diminution of EIN/EIL transcription factor and reduction of the ethylene signalling. These expression data suggest that ethylene and auxin may be involved in floral initiation process in rose although further experiments will be necessary to validate these hypotheses.

Gene expression associated with rose floral development

We harvested six pools of samples corresponding to different flower development stages in *R. chinensis* cv. Old Blush (Figure 1) and compared the transcriptome in successive stages (Figure 2B). We found three distinct groups with common genes (T-test). These groups corresponded to early, mid and late floral development (Figure 2B). A total of 135, 401 and 456 sequences appeared significantly and differentially regulated at least once during early, mid and late flower development stages, respectively.

To validate and evaluate the accuracy of the microarray data, we performed quantitative real-time PCR (qPCR). Twenty four genes were selected from the microarray transcriptomics comparisons based on previous bibliographic reports and/or deregulation levels, then, using qPCR, we further characterized the expression profiles (Figure 3; Figure S1). The correlation between the microarray results and those obtained by qPCR was assessed by calculating the Pearson's product moment correlation coefficient [47,48] (Table S5). Pearson's correlation coefficient was calculated between each pair of fold change as estimated by microarray and qPCR experiments. The statistical significance of each Pearson's correlation coefficient was assessed using the cor.test routine in R. A global correlation coefficient of 0.858 calculated by the average of every gene was observed. These results indicate that our microarrays are able to detect consistently both low and high foldchanges with high accuracy in different experimental conditions (Table S5).

Transcriptome analyses during early flower development

135 genes were differentially expressed at during early floral organogenesis. Among these genes, 46 were found differentially expressed between stages 1+2 and 3+4 and 105 genes were differentially expressed between stages 3+4 and 5 (Table 2 and Table S6). An ACC synthase (AY803737) putative homologue was among the highly up-regulated genes between stages 1+2 and 3+4. In *Arabidopsis*, there are nine ACC synthases, many of which are expressed in the flower [29,30]. The floral organ identity MADS-box encoding genes [31,32,33], such as an *APETALA3* homologue (*RhTM6/MASAKOB3 AB055966*, Figure 3), the *AGAMOUS* ortholog (*RhAG, AB025645*, Figure 3), or the rose *PISTILLATA*

Table 1. Top list of putative floral repressors and activators shared between R. x. wichurana and "Félicité et Perpétue".

		R. x. wichurana		"Félicité et Perpétu	e"
Gene	annotation	Log(ratio) VM2/WM1	Log(ratio) FM/WM2	Log(ratio) VM2/WM1	Log(ratio) FM/WM2
repressors					
EC58630	0,00	-2,77	-0,95	-1,03	-0,71
BQ104485	(Q5NE18) Formate dehydrogenase	-2,75	1,05	-1,14	-
EC589917	(Q3T923) Fra a 1 allergen (Fra a 1-A allergen)	-2,08	-0,34	-4,93	0,20
CF349421	(Q7XHM6) Hypothetical protein OSJNBb0095H08.9	-1,98	1,13	-0,75	-0,29
CF349812	(Q8H7G2) Hypothetical protein (Q8H7G2_ARATH)	-1,97	1,22	-1,00	0,32
EC587235	0,00	-1,81	0,76	-0,84	0,46
EC587845	(Q8L5Z1) Hypothetical protein At1g33810 (Q8L5Z1_ARATH)	-1,79	0,03	-1,65	0,41
CF349438	(O81644) Villin-2 (VILI2_ARATH)	-1,78	2,21	-0,87	0,07
CF349322	(Q1RST0) Peptidase S1 and S6	-1,76	2,40	-2,10	0,01
CF349916	(Q564G6) Galactomannan galactosyltransferase	-1,72	0,60	-1,01	-0,24
EC587239	0,00	-1,71	1,57	-1,68	0,36
BQ105944	ATP-dependent Clp protease ATP-binding subunit	-1,70	1,69	-1,08	-0,13
BQ105308	(Q533V0) Phospholipase D alpha (EC 3.1.4.4)	-1,69	1,85	-0,84	0,31
CF349664	(Q1S2R3) GIGANTEA protein (Q1S2R3_MEDTR)	-1,68	-0,13	-1,27	0,16
BI977439	(Q8L553) SCARECROW transcriptional regulator-like	-1,67	1,13	-1,29	-0,19
EC586479	0,00	-1,66	1,40	-1,24	-0,24
BQ104603	0,00	-1,65	2,31	-1,19	1,00
EC588955	0,00	-1,61	0,19	-1,01	0,67
BQ106662	(O04136) Homeobox protein knotted-1-like 3 (KNAP3))	-1,58	1,09	-1,02	-0,17
CF349422	(Q9SWH0) Plasma membrane proton ATPase	-1,53	1,54	-1,03	0,13
BQ106489	0,00	-1,52	0,98	-2,22	-
EC586088	(Q41695) Pectinacetylesterase precursor	-1,48	1,58	-1,32	0,86
EC588764	(Q2HTG1) GTP-binding signal recognition particle SRP54	-1,48	1,09	-1,26	-0,37
BI977401	(Q2AAC8) Cysteine proteinase	-1,46	0,60	-1,50	0,13
BQ104821	(Q2R3E0) Alpha-mannosidase	-1,46	1,43	-0,74	-0,11
BQ103923	(Q84V96) Aldehyde dehydrogenase 1 precursor	-1,46	0,74	-1,64	0,58
BQ104041	0,00	-1,45	1,98	-1,47	0,43
EC587517	(Q71BZ1) Type-B response regulator (Q71BZ1_CATRO)	-1,42	1,58	-0,70	-
EC588090	0,00	-1,42	-	-2,18	0,48
BQ105490	0,00	-1,37	1,83	-5,92	-0,84
BQ103990	(Q8RWI9) Hypothetical protein At3g21090 (Q8RWI9_ARATH)	-1,36	1,32	- 1,01	-0,26
EC586608	0,00	-1,30	0,91	-0,99	0,38
BI978794	(Q05349) Auxin-repressed 12.5 kDa protein (12KD_FRAAN)	-1,30	0,49	-2,62	-0,80
CF349291	(Q9SGU9) Similar to O-succinylhomoserine sulfhydrylase	-1,26	0,89	-0,96	0,01
EC586448	(Q94BT2) Auxin-induced in root cultures protein precursor	12-1,26	-0,57	-0,77	-0,72
activators					
BI978967	(Q6Z2K3) Putative Avr9/Cf-9 rapidly elicited	1,06	0,27	0,75	-0,04
BI977621	(Q8L5J6) Expansin 3 (Q8L5J6_MALDO)	1,08	-0,50	1,01	0,98
BQ104361	(Q650W6) Putative nucleic acid-binding prot.	1,08	-	1,40	-0,29
EC588171	(Q1SZF1) Allergen V5/Tpx-1 related	1,11	-0,75	1,29	0,79

Table 1. Cont.

		R. x. wichurana		"Félicité et Perpétu	e"
Gene	annotation	Log(ratio) VM2/WM1	Log(ratio) FM/WM2	Log(ratio) VM2/WM1	Log(ratio) FM/WM2
EC586116	0,00	1,12	-1,17	1,14	0,34
EC589388	(Q1SHH7) Auxin responsive SAUR protein	1,14	-0,02	1,52	0,47
BI978946	(Q93Z01) AT5g58730	1,20	-1,03	1,55	-0,17
RoAGL20	(Q7Y137) MADS-box protein PTM5	1,22	-	0,97	0,14
BQ105514	0,00	1,23	-0,74	0,72	-0,02
BI977348	(Q94AQ7) Hypothetical protein At5g11280	1,23	0,50	0,73	-0,47
BQ103904	(Q41696) Cysteine protease precursor	1,25	-2,35	2,55	0,20
EC589855	0,00	1,27	-0,03	0,71	-0,44
BI978115	(Q84W81) Hypothetical protein At5g49800	1,27	0,34	1,85	-0,57
EC586690	Q2QXK7) F-box domain, putative	1,31	-0,91	1,36	-0,02
EC588294	(Q1S0D0) Glyoxalase/bleomycin resistance protein	1,32	-0,46	0,77	1,19
EC588783	(Q9LUC1) Putative protein At3g14740	1,34	0,27	1,19	-0,31
RoAP1a	(Q283Q1) APETALA1 protein	1,38	-0,40	2,06	0,88
EC587486	0,00	1,42	-0,62	1,38	-0,35
BI978732	(P32293) Auxin-induced protein 22A	1,47	-0,36	1,27	1,28
BQ104100	0,00	1,55	-0,94	1,49	1,03
BQ105108	(O65744) GDP dissociation inhibitor	1,63	-3,09	2,13	0,17

Log(ratio) of intensities are represented, italicized numbers represent ratios for which the p-value of the Bonferroni test was higher than 0.05. -: no value could be calculated.

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ortholog (RhPI/MASAKO BP, AB038462), were among the genes whose expression was up-regulated between stages 1+2 and 3+4 or between stages 3+4 and 5. Interestingly, genes that are predicted to have functions in cell wall remodelling, such as putative extracellular lipases (BQ106293, EC586717, EC588243, BI978064, BI977386, BQ105800), xyloglucan endotransglucosylase/hydrolase 2 (XTH2, DQ320658) [34], expansins (BI977621, EC589557), putative pectin esterase (BQ105504) and pectate lyase (BQ103887, BQ105987) were up-regulated between stages 3+4 and 5. This result supports the idea that very active cell wall remodeling coincides with the beginning of organ elongation that occurs mainly at stage 5. A putative gibberellin 2-oxidase (BQ105545) was up-regulated early during flower development. In Arabidopsis, a similar up-regulation of genes implicated in gibberellins metabolism and signaling have been described at early floral development [35,36]. In agreement with previously published data, our microarray analysis suggests that gibberellins are important during early floral development of rose plants [13,37]. Among the genes that showed strong down-regulation between stages 1+2 and 3+4, we found the putative orthologues of PERIANTHIA (PAN), AP1 and SOC1 (AGL20). In Arabidopsis, PAN, AP1 and SOC1 are expressed in the floral meristem, but their expression is down-regulated in the subsequent steps during floral organs differentiation [36,38,39,40], hence in agreement with the observed down-regulation of the rose homologues between flower development stages 1+2 and 3+4.

Early to late floral development transition

Sequences corresponding to 401 genes were detected as differentially regulated between stages 5 and VP. Among these genes, 233 were down-regulated and 168 were up-regulated (see Table 3 for a selection of genes and Table S7 for full list). Genes that exhibit strong similarities to genes involved in carotene, flavonoid and anthocyanin biosynthesis are up-regulated between stages 5 and VP. Among these genes, putative phytoene synthase (BI979026), zeta carotene desaturase (CF349648), lycopene betacyclase (BQ105122) are likely to be involved in carotenoid biosynthesis. The expression of UDP-glucose anthocyanidinoglucosyltransferase (AB201048/*RhGT1*), previously involved in anthocyanin synthesis [41], was strongly up-regulated. A similar strong up-regulation was observed for genes encoding putative phenylalanine ammonia-lyase (BQ105227), chalcone synthase (EC587811), flavonol synthase (AB038247) and anthocyanidin synthase (BI977949) (Figure 3). Altogether, these genes are likely good candidates involved in anthocyanins biosynthesis in rose petals.

Interestingly, genes predicted to encode five putative cyclins (EC586028, EC586517, EC587578, EC588351, and EC588489) and a putative cyclin dependent kinase (EC589228) are strongly down-regulated during floral organ morphogenesis. This down-regulation may reflect the transition from mitotic growth to post-mitotic growth where floral organs grow through cell expansion. Recently, Vanneste *et al.* showed that the transcriptional down-regulation of A2 type cyclins is a direct link between developmental programming and cell-cycle exit in *Arabidopsis thaliana* [42].

Fifteen genes encoding putative transcription factors were upregulated, while nine were down-regulated. Among the upregulated transcription factors, we found the putative orthologue of *SHP* (AB025643) [32] and a putative NAC domain protein (BI978992, Figure 3). BI978992 is homologous to *Arabidopsis NAC2*, a gene expressed in ovule integuments. The differential expression of *NAC2* between stages 5 and visible petals (VP) suggests its putatively conserved function with the *Arabidopsis NAC2*. Three putative MYB transcription factors were also up-







Figure 3. Real time quantitative RT-PCR (qPCR) analysis of six selected differentially expressed genes during rose floral organogenesis, floral opening and senescence in *R. chinensis* cv. Old Blush. qPCR data (black histograms) are compared to the microarray hybridization data (white histograms). Microarray data is presented regardless of Bonferroni test success. Each pair of histograms represent successive comparisons between floral development stages 1+2, 3+4, 5, visible petals (VP), open flower (OF) and senescing flowers (SF). doi:10.1371/journal.pone.0028455.g003

regulated (CF349636, BQ104100 and BI978095, Figure 3). These rose MYBs may be involved in organ elongation, as they share about 67% protein sequence similarity with AtMYB21, known to be involved in gibberellins/jasmonate-mediated control of stamen filament elongation [43].

Late floral development

456 genes were differentially regulated at least once during the late phases of floral development, i.e. from visible petal (VP) stage to senescent flower (SF) stage. Most of these genes showed similar expression pattern when we compared stages VP to OF (open

flower) or stages VP to SF (See Table 4 for top list, and Table S8 for full data). This result indicates that the transcriptome becomes less dynamic at senescence stages and thus not so many differences are detected when comparing samples OF and SF to the VP sample. Gene ontology analysis showed that among the up-regulated genes, the three GO terms chlorophyll catabolic process, heterocycle catabolic process and cellular nitrogen compound catabolic process were significantly overrepresented as compared to the whole annotated set; the four GO terms nucleus, macromolecule biosynthetic process, intracellular non-membrane-bounded organelle and ribonucleoprotein complex were

Table 2. List of selected floral organogenesis associated genes in R. chinensis cv Old Blush.

		R. chinensis cv Old Blush	
Gene	annotation	Stages 3+4 vs 1+2	Stages 5 vs 3+4
AY803737	Rosa hybrid cultivar 1-aminocyclopropane-1-carboxylase synthase 2 (ACS2)	2,99	-1,21
AB055966	Rosa rugosa MASAKO B3 mRNA for MADS-box protein,	2,67	1,04
AB025645	Rosa rugosa MASAKO C2 mRNA for MADS-box protein,	2,94	1,37
CF349463	(Q1S9M3) Lipase, active site (Q1S9M3_MEDTR)	2,68	-
BI978064	(Q9M8Y5) Putative GDSL-motif lipase/acylhydrolase (Q9M8Y5_ARATH)	2,10	1,17
BI977386	(Q9M8Y5) Putative GDSL-motif lipase/acylhydrolase (Q9M8Y5_ARATH)	1,99	1,08
EC586717	(Q1S3U7) Lipolytic enzyme, GDS-L (Q1S3U7_MEDTR)	1,69	1,16
BQ105800	(Q1SAY6) Lipolytic enzyme, GDSL (Q1SAY6_MEDTR)	2,76	0,91
DQ320658	Rosa×borboniana xyloglucan endotransglucosylase/hydrolase 2 (Xth2)	2,55	0,74
BI977621	(Q8L5J6) Expansin 3 (Q8L5J6_MALDO)	-0,89	1,26
EC589557	(Q9SBT1) Expansin (Q9SBT1_FRAAN)	0,48	1,20
BQ105987	(Q94FT6) Pectate lyase B (Fragment) (Q94FT6_FRAAN)	0,75	1,27
BQ103887	(Q52PJ2) Ripening-related pectate lyase (Q52PJ2_MANIN)	1,21	1,11
BQ105504	(Q7X9B1) Pectinesterase (EC 3.1.1.11) (Q7X9B1_FRAAN)	1,44	1,35
BQ105545	(Q4W8C3) Gibberellin 2-oxidase (Q4W8C3_PHAAN)	0,42	-1,35
RoPAN	(Q9SX27) Putative bZIP transcription factor, PERIANTHIA (Q9SX27_ARATH)	- 1,94	-2,08
RoAGL20	(Q7Y137) POPTM (Q7Y137) MADS-box protein PTM5	-2,77	-0,03
RoAP1b	(Q2XUP6) MADS-box protein	-0,98	-3,15

Log(ratio) of intensities are represented, italicized numbers represent ratios for which the p-value of the Bonferroni test was higher than 0.05. doi:10.1371/journal.pone.0028455.t002

underrepresented. We could identify two genes encoding staygreen protein homologues (BI978267 and BQ106457) that are strongly up-regulated upon petal elongation and remain highly expressed throughout the final petal senescing process. Stay-green proteins have a major role in chlorophyll and photosynthetic pigments degradation and have been repeatedly described to be associated with the processes of fruit ripening and organ senescence [44]. Surprisingly, no gene related to ethylene biosynthesis or signaling was detected as differentially expressed during late floral development. However the RbXTH1 and RbEXPA1 genes, both induced during ethylene-triggered and field abscission [34,45], were strongly up-regulated between VP and OF stages and remained as such in senescing flowers. Among the down-regulated genes, the two GO terms protein metabolic process and plasma membrane were underrepresented as compared to the whole set (whole microarray GO terms) and the eight GO terms acyltransferase activity, acyl-carrier-protein biosynthetic process, acyl carrier activity, cellular carbohydrate metabolic process, polysaccharide metabolic process, fatty acid biosynthetic process, lipase activity and defense response to fungus were overrepresented (Table 5). The enrichment in the latter set may represent a slowdown of general metabolic pathways at the onset of flower senescence. Similar results were reported in A. thaliana during organs senescence where a down-regulation of the photosynthetic machinery accompanied by a reduction in expression of many cell wall biosynthetic genes reflecting a cessation of growth during senescence [46].

Conclusions

We established a calendar of the floral initiation and development for the rose and developed a rose microarray that harbors sequence from genes expressed during the floral transition and whole floral development process in *Rosa sp*, from initiation up

to senescing flowers. This microarray and the floral development calendar were successfully used to identify genes whose expression correlated with different flower development stages. These multiple datasets represent an extensive study of rose floral development. This resource can be helpful to select candidate genes potentially involved in different horticultural traits, such as flowering, floral architecture, scent production and emission, senescence and abscission. We used the microarray developed herein to identify genes whose expression is associated with some of these rose important traits, such as flower initiation, development and senescence. Rosa1_Affyarray harbors sequences from ESTs found in petals of different rose genotypes [5,14] (http://urgi.versailles. inra.fr/GnpSeq) and thus may be helpful to identify genes associated with other rose traits such as scent biosynthesis and/ or emission genes. The rose is among the species that exhibit the highest scent complexity [47-48] [12] and some scent biosynthesis pathways are unique to the rose or not vet identified in other model species including other members of the Rosaceae genus [11,49]. OTLs have been identified to be associated to several important traits of the rose [50]. However, the heterozygous genome of the rose complicates the breeding programs to select for several traits simultaneously. The identification of genes whose expression correlates with important ornamental traits can facilitate and accelerate candidate gene identification for rose breeding by marker assisted selection or genomic selection. For example, this dataset can provide researchers with a useful resource on the expression of candidate genes within a given mapping interval. Furthermore, the rapidly progressing high throughput sequencing technologies should allow the generation of precise genetic maps for the rose that could be combined to refined transcriptomics approaches to identify the genes responsible for important horticultural traits in the rose, and allow subsequent marker-assisted selection.

Table 3. List of selected genes associated with early to late flower development in R. chinensis cv Old Blush.

		R. chinensis cv Old Blush
Gene	annotation	5 vs PA
BI978095	(P93474) Myb26	8,00
BI978992	(Q50J79) NAM-like protein	5,28
AB038247	Rosa hybrid cultivar 'Kardinal' FLS mRNA for flavonol synthase	4,67
BQ105122	(Q9SEA0) Lycopene beta-cyclase	4,30
EC587811	(Q84UT9) Chalcone synthase	3,27
BQ104100	MYB domain class transcription factor	3,01
AB025643	Rosa rugosa MASAKO D1 mRNA for MADS-box protein.	3,00
CF349648	(Q5W5X6) Zeta-carotene desaturase ZDS2	2,97
BI979026	(Q2VEY1) Putative phytoene synthase	2,89
BI977949	(Q5UL09) Anthocyanidin synthase	2,18
AB201048	RhGT1 UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase,	2,18
CF349636	(Q9ATD1) GHMYB9	2,12
BQ105227	(Q9M567) Phenylalanine ammonia-lyase 2	2,11
EC586028	(Q9SNV1) Cyclin D3a (Fragment)	-2,12
AB201051	RhGT4 mRNA UDP-glucose: flavonol 3-O-glucosyltransferase	-2,18
EC587392	(Q8S342) Putative anthocyanidine rhamnosyl-transferase	-2,45
EC587578	(Q6T2Z6) Cyclin d3	-3,83
EC586734	(Q08733) Aquaporin PIP1.3	-4,52
RhCyc2	(Q9SBQ4) CYCB1-1 protein	-4,65
EC588351	(Q9SBQ4) CYCB1-1 protein	-4,72
EC58848	(P93557) Mitotic cyclin	-4,77
EC589228	(Q94EX2) At1g76540/cyclin dependent kinase	- 5,05
EC586517	(Q4JF78) Cyclin-dependent kinase B	-5,26

Log(ratio) of intensities are represented, for all ratios the p-value of the Bonferroni test was lower than 0.05. doi:10.1371/journal.pone.0028455.t003

Materials and Methods

Plant material

R. wichurana was obtained from 'Jardin de Bagatelle' (Paris, France) and *R. x hybrida* cv. Félicité et Perpétue from the Loubert Nursery (Rosier sur Loire, France). Plants were grown outdoors on their own roots as previously described [13]. In spring, at different time points (see results), terminal parts of the growing shoot were harvested and partly dissected (removal of young leaves). *R. chinensis* cv. Old Blush was propagated by cuttings from the Lyon Botanical Garden. Plants were grown in the greenhouse with 16 h/8 h day/night and 25°C/14°C day/night temperature. No specific permits were required for the described filed studies, no specific permissions were required for these locations, the location is not privately owned or protected, and the field studies did not involve endangered or protected species.

Light microscopy and SEM imaging of meristems and early flower development

Samples were dissected under a binocular stereomicroscope and then fixed in 4% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C under vacuum. Samples were dehydrated in a graded ethanol series and embedded in Technovit 7100 [51]. Sections of 1.5 to 2.0 μ m (Leica RM 2165 microtome) were stained with toluidine blue and examined under an Olympus BH2-RFC microscope coupled to a 3CCD Sony camera.

For scanning electron microscopy, terminal part of the shoot was carefully dissected. After a fixation in 4% glutaraldehyde (v/v), followed by post-fixation with osmium tetroxide, the sample was dehydrated in a graded alcohol series and in acetone. Dehydration was completed by critical point drying. Sample were then coated with gold (MED 020 BALTEC) and observed with a JEOL JSM-63017 scanning electron microscope.

RNA samples preparation

Two independent biological replicates were produced for each samples at different stages. For each biological repetition and each point, RNA samples were obtained by pooling vegetative or floral tissue from at least five different plants. For *R. chinensis* cv. Old Blush samples, meristems or flowers were dissected and collected individually on plants at developmental growth stages, cultivated in greenhouse conditions as previously described [55]. For *R. wichurana* and *R. x hybrida* cv. Félicité et Perpétue, RNA was extracted from non-dissected buds, including either the vegetative meristem and its surrounding leaves or the pre-floral/floral meristem and its surrounding leaves and bracts.Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) according to the supplier's instructions.

AFFYMETRIX Array hybridization

RNA samples were checked for their integrity on The Agilent 2100 bioanalyzer according to the Agilent Technologies (Waldbroon, Germany).

Table 4. List of selected floral maturation and senescence associated genes in R. chinensis cv Old Blush.

		R. chinensis cv C	ld Blush
Gene	annotation	PA vs FE	PA vs FS
BI977502	Brassinosteroid-regulated protein BRU1 precursor	8,82	8,79
BI978598	Early light-induced protein	8,38	7,99
EC587309	0,00	7,70	5,82
BI977376	Putative zinc finger protein At1g68190	7,35	7,13
BI978143	0,00	6,61	7,00
EC587486	0,00	6,31	4,81
BI978750	0,00	6,30	5,55
BI978596	Hypothetical protein	6,29	6,32
BQ104828	0,00	6,24	5,51
BQ105724	0,00	6,09	5,65
EC586975	Glycosyltransferase NTGT5a	6,07	6,28
BQ106572	0,00	6,02	5,87
BI977926	18.5 kDa class I heat shock protein	5,83	-
EC588495	0,00	5,60	5,54
BI978508	Hypothetical protein	5,51	-
BO104475	0.00	5.50	6.25
BO103870	0.00	5.26	5.12
BI977873	Hypothetical protein At5g63130	5.25	5.18
BO103973	Tryptophan synthase alpha chain	5.24	4.61
BI977634		5.21	5.20
BO105490	0.00	5,07	5,85
BQ106477	Protein WUSCHEL-like	5,03	5,54
BQ106330	0.00	5,02	5,22
BQ106091	Hypothetical protein At2g42570	4.91	4.52
BI977302	Bzip transcription factor	4.82	4.83
BI978926	ΑΤ5α11580	4.81	5.46
CE349316	Putative NADH dehydrogenase	4.81	5,01
EC 589818	Putative calmodulin-related protein	4.80	-
BI978132		4 74	4 55
BO105726	Expansin-like protein	4 73	3 90
BQ103720	0.00	4 68	4.81
EC 586683	0.00	4 68	5 52
DO320657	Rosa x horboniana expansin protein (ExpA1) mRNA	4 17	4 38
EC589229	Probable vyloglucan endotransglucosylase/bydrolase protein 8 precursor	3 20	-
BI978267	Senescence-inducible chloronlast stay-green protein 1	2 71	2.42
BO106457	Senescence-inducible chloroplast stay-green protein 2	-	2 34
BQ100137	Pertate lyase	-404	-6.18
FC 588897		-4.04	-4.86
EC588483		-4.06	-3.72
EC587152		-4.08	-4.46
EC589569	Serine carbovupentidase putative	-4.10	-3.48
EC586717		-4.13	-2.59
EC587284	RNA-binding region RNP-1	-4.15	-342
BI977461		-4.21	-4.40
EC586015	Cold-regulated LTCOR12	-4.22	-4.44
RI978116		-4.24	
BI977349	Hypothetical protein At5a11280	-4.27	-4.99
BO106043		-4 27	-4.01
EC580137		-4.33	-4.07
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Table 4. Cont.

		R. chinensis cv O	ld Blush	
Gene	annotation	PA vs FE	PA vs FS	
EC587785	0,00	-4,37	-3,91	
EC586984	Putative beta-expansin	-4,38	-4,99	
CF349422	Plasma membrane proton ATPase	-4,41	-3,64	
BI977751	0,00	-4,49	-4,43	
EC589098	Phosphoethanolamine N-methyltransferase 1	-4,49	-5,79	
BQ106293	GDSL-motif lipase/hydrolase-like protein	-4,51	-6,32	
CF349724	Glucosyltransferase-like protein	-4,74	-4,10	
BQ106328	Pathogenesis-related transcriptional factor and ERF	-5,10	-	
EC586884	Proline-rich protein APG-like	-5,11	-3,72	
CF349712	Senescence-inducible gene protein	-5,12	-5,47	
BI978135	Plant lipid transfer protein/Par allergen	-5,16	-7,04	
CF349692	Putative alcohol oxidase	-5,17	-6,66	
EC588080	Hypothetical protein (At2g35760/T20F21.5)	-5,32	-5,20	
BI977262	Putative lipase	-5,48	-6,04	
CF349791	Globulin-like protein (Fragment)	-5,67	-5,53	
AB121046	phloroglucinol O-methyltransferase, complete cds	-6,13	-5,06	
BI978206	0,00	-8,51	-	
BI978064	Putative GDSL-motif lipase/acylhydrolase	-10,47	-10,51	
BI977386	Putative GDSL-motif lipase/acylhydrolase	-11,60	-11,42	

Log(ratio) of intensities are represented, for all ratios the p-value of the Bonferroni test was lower than 0.05.

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Two µg of total RNA were used to synthesize biotin-labeled cRNAs with the One-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). Superscript II reverse transcriptase and T7-oligo (dT) primers were used to synthesize the single strand of cDNA at 42°C during 1 hour followed by the synthesis of the double stranded cDNA by using DNA ligase, DNA polymerase I and RNaseH during 2 hours at 16°C. Clean up of the double-stranded cDNA was performed with Sample Cleanup Module (Affymetrix) followed by in vitro transcription (IVT) in presence of biotin-labeled UTP using GeneChip® IVT labelling Kit (Affymetrix). Quantity of the labelled-cRNA with RiboGreen® RNA Quantification Reagent (Turner Biosystems, Sunnyvale, CA) was determined after cleanup by the Sample Cleanup Module (Affymetrix). Fragmentation of 10 µg of labelled-cRNA was carried out for 35 minutes at 94°C, followed by hybridization during 16 hours at 45°C to Affymetrix GeneChip[®] Rosal Genome Array representing approximately 4869 genes. After hybridization, the arrays were washed with 2 different buffers (stringent: 6× SSPE, 0.01% Tween-20 and nonstringent: 100 mM MES, 0.1 M [Na+], 0.01% Tween-20) and stained with a complex solution including Streptavidin R-Phycoerythrin conjugate (Invitrogen/molecular probes, Carlsbad, CA) and anti Streptavidin biotinylated antibody (Vectors laboratories, Burlingame, CA). The washing and staining steps were performed in a GeneChip® Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip® Rosa1 Genome Arrays were finally scanned with the GeneChip® Scanner 3000 7G piloted by the GeneChip® Operating Software (GCOS).

Statistical Analysis of Microarray Data

The data were normalized with the gcrma algorithm [52], available in the Bioconductor package [53]. To determine differen-

tially expressed genes, we performed a usual two group t-test that assumes equal variance between groups. The variance of the gene expression per group is a homoscedastic variance, where genes displaying extremes of variance (too small or too large) were excluded. The raw P values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate (FWER) [54]. A gene is declared differentially expressed if the Bonferroni P-Value is less than 0.05.

Data Deposition

All this steps were performed on Affymetrix plateform at INRA-URGV, Evry. The raw. CEL files were imported in R software for data analysis. All raw and normalized data are available through the CATdb database (AFFY_PetalDvt_Lyon_Rose, [55]) and from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) [56], accession number GSE18357.

Validation of genes expression using quantitative realtime PCR

Only genes that were involved in floral development were analyzed for microarray data validation. One microgram total RNA (treated with DNAse) was used in a reverse transcription assay with RevertAid M-MuLV Reverse Transcriptase (Fermentas, Burlington, Ontario). Target cDNAs were quantified by qPCR using FastStart universal SYBR green master (Roche, Basel, Switzerland) on a Step-OnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA USA). Expression levels were normalized with *RhaTubuline*, *RhGAPDH* and *RhEF1a* reference genes. These genes were validated as reference genes using the GeNorm application [57]. Three independent biological replicates (pools of dissected flowers from at least 5 different plants) were used for each experiment and two qPCR technical replicates were **Table 5.** Gossip analysis of GO terms enrichment in late flower development dataset (genes that are differentially expressed at least once during floral maturation and senescence).

	GO Term	Name	FDR	FWER	single test p-Value	# in test group	# in reference group	# non annoted test	# non annoted reference group	Over/Under
Late floral development upregulated genes	GO:0005634	nucleus	0.0	0.0	0.012	0	107	56	1290	under
	GO:0009059	macromolecule biosynthetic process	0.0	0.0	0.028	0	88	56	1309	under
	GO:0043232	intracellular non- membrane-bounded organelle	0.0	0.0	0.030	0	86	56	1311	under
	GO:0030529	ribonucleoprotein complex	0.0	0.0	0.030	0	84	56	1313	under
	GO:0015996	chlorophyll catabolic process	0.008	0.008	5.43E-5	3	0	53	1397	over
	GO:0046700	heterocycle catabolic process	0.028	0.062	5.13E-4	3	2	53	1395	over
	GO:0044270	cellular nitrogen compound catabolic process	0.028	0.062	5.13E-4	3	2	53	1395	over
Late floral development downregulated genes	GO:0019538	protein metabolic process	0.0	0.0	0.009	5	225	73	1150	under
	GO:0005886	plasma membrane	0.012	0.006	0.013	4	192	74	1183	under
	GO:0008415	acyltransferase activity	0.016	0.025	1.62E-4	7	17	71	1358	over
	GO:0042967	acyl-carrier-protein biosynthetic process	0.016	0.028	2.15E-4	7	18	71	1357	over
	GO:000036	acyl carrier activity	0.027	0.079	5.73E-4	3	1	75	1374	over
	GO:0044262	cellular carbohydrate metabolic process	0.027	0.084	6.73E-4	15	99	63	1276	over
	GO:0005976	polysaccharide metabolic process	0.027	0.086	7.08E-4	10	48	68	1327	over

The reference group that was used corresponds to the full annotated sequences (sequences with GO terms) of the microarray. doi:10.1371/journal.pone.0028455.t005

performed for each biological replicate. Primer sequences are available in Table S9. The correlation between the microarray results, and those obtained by qPCR was assessed by calculating the Pearson's product moment correlation coefficient [58,59].

Supporting Information

Figure S1 Real time quantitative RT-PCR (qPCR) analysis of 18 selected differentially expressed genes during rose floral organogenesis and senescence in *R. chinensis* cv Old Blush.

(TIFF)

Table S1 $\,$ Full array sequences annotation and ontology. $\rm (XLSX)$

Table S2Genes differentially expressed during floralinitiation in R. wichurana.(XLSX)

Table S3Genes differentially expressed during floralinitiation in R. x hybrida cv. Félicité et Perpétue.(XLSX)

Table S4 List of genes repressed (A) or activated (B) during flower initiation. (XLSX)

Table S5 Microarray and qRT-PCR results of 25 selected genes with their replicate-level Pearson correlation.

(DOCX)

Table S6Genes differentially expressed during earlyfloral organogenesis in R. chinensis cv. Old Blush.(XLSX)

Table S7Genes differentially expressed during floralorgan elongation in R. chinensis cv. Old Blush.(XLSX)

Table S8Genes differentially expressed during floweropening and senescence in R. chinensis cv. Old Blush.(XLSX)

Table S9Primers used in this study.(DOC)

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Author Contributions

Conceived and designed the experiments: MB AD OR. Performed the experiments: AD AR OR SB AC MM YP SHY JJ TT VB MLMM SJ JPR

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PV MLB FF. Analyzed the data: AD OR SB MLMM PV MLB FF MB. Contributed reagents/materials/analysis tools: FL. Wrote the paper: AD MB.

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ORIGINAL PAPER

A survey of flowering genes reveals the role of gibberellins in floral control in rose

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Abstract Exhaustive studies on flowering control in annual plants have provided a framework for exploring this process in other plant species, especially in perennials for which little molecular data are currently available. Rose is a woody perennial plant with a particular flowering strategy-recurrent blooming, which is controlled by a recessive locus (RB). Gibberellins (GA) inhibit flowering only in non-recurrent roses. Moreover, the GA content varies during the flowering process and between recurrent and nonrecurrent rose. Only a few rose genes potentially involved in flowering have been described, i.e. homologues of ABC model genes and floral genes from EST screening. In this study, we gained new information on the molecular basis of rose flowering: date of flowering and recurrent blooming. Based on a candidate gene strategy, we isolated genes that have similarities with genes known to be involved in floral control in Arabidopsis (GA pathway, floral repressors and integrators). Candidate genes were mapped on a segregating population, gene expression was studied in different organs and transcript abundance was monitored in growing

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A. Remay · D. Lalanne · T. Thouroude · F. Le Couviour · L. Hibrand-Saint Oyant · F. Foucher (⊠) INRA d'Angers Nantes, IFR 149 Quasav, UMR 1259 GenHort, BP60057, 49071 Beaucouzé cedex, France e-mail: fabrice.foucher@angers.inra.fr shoot apices. Twenty-five genes were studied. *RoFT*, *RoAP1* and *RoLFY* are proposed to be good floral markers. *RoSPY* and *RB* co-localized in our segregating population. GA metabolism genes were found to be regulated during floral transition. Furthermore, GA signalling genes were differentially regulated between a non-recurrent rose and its recurrent mutant. We propose that flowering gene networks are conserved between *Arabidopsis* and rose. The GA pathway appears to be a key regulator of flowering in rose. We postulate that GA metabolism is involved in floral initiation and GA signalling might be responsible for the recurrent flowering character.

Introduction

Flowering, which is the vegetative to reproductive transition phase, is a critical developmental stage in a plant's life. In monocarpic plants, this transition happens only once, whereas polycarpics have several flowering cycles throughout their life. Monocarpics have four growth phases. First, the juvenile phase followed by the adult vegetative phase, then floral transition leads to the floral phase, ending with senescence (Baurle and Dean 2006). In comparison, the polycarpic life cycle is more complex. Flowering represents only a transitory state before new vegetative growth in polycarpic plants-they must maintain an equilibrium between vegetative and reproductive development throughout their life. Flowering generally occurs once a year. However, some polycarpics can reproduce several times a year. This process is known as recurrent blooming in rose or day neutral in strawberry, for instance (Battey et al. 1998).

Flowering has been widely studied in monocarpic plants at the genetic and molecular levels. In the model plant *Arabidopsis thaliana*, four different pathways controlling floral

initiation have been described: photoperiod and light quality, the autonomous pathway, vernalization and gibberellins (GA; reviewed in Araki 2001; Boss et al. 2004; Mouradov et al. 2002; Simpson and Dean 2002). These four pathways converge to activate the floral integrators, FT (FLOWER-ING LOCUS T; Kardailsky et al. 1999; Kobayashi et al. 1999) and SOC1 (SUPRESSOR OF CONSTANS; Onouchi et al. 2000), and repression of a central repressor, FLC (FLOWERING LOCUS C; Michaels and Amasino 1999; Sheldon et al. 2000b). In turn, the floral integrators activate the meristem identity genes, LFY and AP1 (reviewed in Araki 2001; Simpson and Dean 2002). Organ identity genes are the targets of these floral integrators and meristem identity genes. These genes, known as ABC model genes, are in charge of floral development (Jack 2001; Weigel and Meyerowitz 1994).

Plants have to perceive and respond to photoperiod and light quality. Light is perceived by phyto- and cryptochromes (Quail 2002). Changes in daylength are detected by the intrinsic circadian system, which in turn under long day conditions, activates the transcription factor CON-STANS (Suarez-Lopez et al. 2001). *FT* and *SOC1* are direct targets of the photoperiod and light quality (Onouchi et al. 2000; Samach et al. 2000). CO induces *FT* in the leaves (Samach et al. 2000). FT protein moves from leaves to the shoot apical meristem (SAM) (Corbesier et al. 2007) and interacts with the FD transcription factor that is only expressed in SAM (Abe et al. 2005; Wigge et al. 2005). *FT* activates flowering and is a primary candidate for encoding florigen (Zeevaart 2008).

FT and SOC1 are subjected to the repression of FLC. FLC encodes a MADS domain protein that acts as a repressor of flowering (Michaels and Amasino 1999). This repressor is regulated both by the autonomous pathway (Rouse et al. 2002) and vernalization (Sheldon et al. 2000a). Autonomous pathway genes were first described thanks to mutants that flowered late under all photoperiods (Koornneef et al. 1991). It involves seven genes, as reviewed in Simpson (2004), which prevent the accumulation of FLC mRNA. Vernalization involves genes such as VRN1, VRN2 and VIN3 (Gendall et al. 2001; Levy et al. 2002; Sung and Amasino 2004). These genes quantitatively act in response to cold treatment in an epigenetic manner, principally by downregulating FLC expression (Bastow et al. 2004; Sheldon et al. 2006). Other different repressors are also involved in floral initiation control. Unlike FT, TFL1 is a repressor of floral initiation (Bradley et al. 1997). Moreover, TFL1 may play a role in inflorescence meristem identity by regulating the flower indeterminacy (Ratcliffe et al. 1998). The EMBRYONIC FLOWER genes, i.e. EMF1 and EMF2, maintain vegetative development and repress reproductive growth as well (Moon et al. 2003b).

Finally, the GA pathway promotes flowering in Arabidopsis during short days (SD) (Wilson et al. 1992) by the activation of *LFY* and *SOC1* (Blazquez and Weigel 2000; Eriksson et al. 2006; Moon et al. 2003a). GA metabolism principally involves GA oxidase enzymes that are encoded by multigenic family genes (reviewed in Hedden and Phillips 2000). GA20OX and GA3OX are the enzymes responsible for active GA synthesis; GA2OX also plays a key role by converting active into inactive GA (Sakamoto et al. 2004). GA signalling operates as de-repressible system that is moderated by DELLA-proteins (Fleet and Sun 2005). DELLA-proteins are represented by five proteins in Arabidopsis: RGA, GAI, RGL1, RGL2 and RGL3 (Ikeda et al. 2003). DELLA-proteins are activated by SPINDLY (Silverstone et al. 2007). In the presence of active GA, its receptor, GID1 (GA INSENSITIVE DWARF1), recruits SLEEPY and forms a complex with DELLA (McGinnis et al. 2003; Nakajima et al. 2006). Consequently, DELLA protein is degraded via the ubiquitin-proteasome (Sun and Gubler 2004). Then, the system is derepressed and the de facto plant responds to GA.

The unravelled gene network in Arabidopsis for the control of floral transition offers a broad framework for studying flowering in other plants. The network is mainly conserved in monocarpic plants such as rice, but some genes exhibit different regulations (Izawa et al. 2003). In perennials, different studies have shown that homologous genes are also implicated in flowering control. In poplar, homologues of FT are involved in floral initiation (Igasaki et al. 2008). However, a new role has been demonstrated in juvenility and seasonal flowering, which are perennial specific traits (Hsu et al. 2006). In tomato, SFT and SP are described as being the orthologues of the Arabidopsis FT and TFL1 genes, respectively (Lifschitz et al. 2006; Pnueli et al. 1998). SFT and SP interact to control flowering in the sympodial shoot system of tomato (Lifschitz and Eshed 2006). In grass, two sequential signals regulate floral initiation (McDaniel and Hartnett 1996). Cold exposure makes plants to respond to GA, and LD induces GA synthesis, leading to floral transition (McMillan et al. 2005). Finally, in grapevine, GA inhibits floral meristem production (Boss and Thomas 2002). These few examples show that flowering genes are conserved within plants but functions or regulation of these genes can vary between species.

Although the whole sequenced genome of grapevine (Jaillon et al. 2007) and poplar (Tuskan et al. 2006) are now sources of knowledge for flowering in polycarpic plants, a lot remains to be unravelled. In rose, the top-ranking ornamental plant produced worldwide, flowering has been studied at the physiological level in modern recurrent cultivars of cut flower roses (Al-Humaid 2003; Chakradhar and Khiratkar 2004; Horridge and Cockshull 1974). In recurrent rose, flowering is self-inductive, i.e. environmental triggers

are not required to initiate flowering (Havely 1972). In nonrecurrent rose, flowering is under environmental control via vernalization. (Foucher F., personal communication). As in other woody plants, GA inhibits flowering in rose (Roberts et al. 1999; Zeevaart 1983). Exogenous application of GA inhibits flowering in non-recurrent rose, whereas it has no effect on flowering in recurrent rose (Roberts et al. 1999). Moreover, the analysis of GA content in a non-recurrent rose and its spontaneous recurrent mutant showed that the GA concentration varies during floral transition and within the mutant (Roberts et al. 1999). Little information on genetic and molecular flowering control is currently available. The recurrent blooming trait is inherited as a single recessive gene, i.e. RB for RECURRENT BLOOMING (Crespel et al. 2002; Semeniuk 1971). Recently, a QTL for the flowering date was shown to be located in the vicinity of the *RB* locus (Hibrand-Saint Oyant et al. 2008).

Only a few genes have been characterized in rose. These genes are potentially involved in floral control and development. A candidate gene approach has been implemented and led to the isolation of ABC model genes (Hibino et al. 2006; Kitahara et al. 2001; Kitahara and Matsumoto 2000). In a previous study, we sequenced ESTs and thus identified new genes with significant similarities to floral genes (Foucher et al. 2008).

The aim of this study was to explore the molecular basis of rose flowering and more particularly the molecular control of recurrent blooming. According to our knowledge on flowering in rose, we postulate that the GA pathway and floral inhibitors might be key factors. We thus used degenerate primers to isolate genes involved in floral repression, GA signalling and metabolism and floral integrator activation. Genetic mapping and gene expression analysis were performed for genetic and molecular characterization. We propose that GA is a key hormone in the control of flowering in rose; GA metabolism seems to be involved in floral initiation and GA signalling might play a role in recurrent blooming.

Materials and methods

Plant material

Rosa hybrida cv Félicité&Perpétue (FP) and *Rosa hybrida* cv Little White Pet (LWP) were obtained from the Loubert nursery (http://www.rosesloubert.com, Les Rosiers sur Loire, France). *Rosa hybrida* cv Little White Pet resulted from a spontaneous vegetative mutation of *Rosa hybrida* cv Félicité&Perpétue (Lewis 1994). LWP is a recurrent blooming, sterile dwarf rose whereas FP is a non-recurrent, fertile climbing rose (Lewis 1994). A segregating population (HW) of 91 interspecific hybrids derived from a cross

between H190 and a *Rosa wichurana* (Rw) hybrid was used as the mapping population (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008). All of these rose genotypes are diploid. Plants were outdoor grown on their own roots on KLASMANN RHP 15[®] (provided by RIPPERT) substrate in 5 l pots at INRA in Angers, France, and fertilized with LD10[®] (provided by COMPO France SAS).

Growth measurement and sampling

In spring, terminal parts of growing shoots of FP and LWP were regularly taken off at different developmental stages: from lateral bud outgrowth to the floral bud stage. After the first flowering, plants were pruned (beginning of July), then new shoots arose from buds. Autumn samples were harvested when two leaves were fully opened: in FP, shoots remained vegetative (non-recurrent genotype) whereas LWP shoots flowered rapidly (recurrent genotype). Developmental stages were defined as the number of visible leaves per new shoot. Morphological observations were performed using a binocular microscope to detect floral initiation. Floral initiation corresponds to the first morphological changes leading to an inflorescence meristem according to Foucher et al. (2008). Each sample corresponded to the three most distal buds from five plants per genotype.

Isolation of candidate genes: degenerate primer strategy

To design degenerate primers, conserved domains were identified from protein sequence alignments using the BLOCKMAKER online application (http://bioinformatics.weizmann.ac.il/blocks/blockmkr/www/make_blocks.html). Based on these conserved domains, CODEHOP was used to design degenerate primers (Rose et al. 2003).

All primers used for gene cloning are listed in Table S1. For a few genes, full-length cDNAs were obtained using 3' and 5' rapid amplification cDNA ends (Jain et al. 1992) according the manufacturer's recommendations (Clontech, Moutain View, USA).

Sequencing, database searches, alignments and phylogenetic analysis

Sequencing was subcontracted to Genome Express (Grenoble, France).

Sequences of putative rose homologue genes were blasted against the *Arabidopsis* genome (The Arabidopsis Information Resources; http://www.arabidopsis.org) using tBLASTx and tBLASTn searches. Validated sequences were translated (http://bioinfo.hku.hk/services/analyseq/ cgi-bin/traduc_in.pl) and protein alignments were performed with MultAlin online software (Corpet 1988). CLUSTALW (Thompson et al. 1994) and TreeViewX Version 0.5.0 by Roderic D. M. Page (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) were used for phylogenetic analyses and phylogenetic tree edition, respectively.

DNA and RNA extraction

Genomic DNA was isolated from young leaves of rose using the NucleoSpin[®] Plant kit (Macherey-Nagel) according to the manufacturer's recommendations.

Total RNA isolations were performed on growing apices from FP, LWP and Rw. Tissues were ground in liquid nitrogen with 10% (w/w) PVP40 (PolyVinylPyrrolydone), and total RNA was extracted using the Nucleo-Spin[®] RNA plant kit (Macherey-Nagel) according to the manufacturer's recommendations. Nucleic acids were quantified using a Nanodrop (Nanodrop Technologies Inc., Wilmington, USA) and their quality was checked either by electrophoresis on agarose gel or using the Agilent capillary electrophoresis system (Agilent Technologies).

Reverse transcription (RT)

Three microgram of total RNA was DNase-treated for 15 min at 25°C with 1 U of RQ1 RNase-free DNase (Promega, Madison, USA) in a final volume of 10 μ l containing the appropriate buffer followed by enzyme inactivation by adding 2 mM of EGTA and incubation for 10 min at 65°C. RNAs were denatured for 5 min at 70°C with 0.5 μ g of oligo(dT)15 (Promega) and then subjected to reverse transcription with 200 U of MMLV-RT (Promega), 0.5 mM of each dNTP, 1 U of RNasin[®] Ribonuclease Inhibitor (Promega) in a final volume of 25 μ l for 1 h at 42°C. After RT, the reaction volume was adjusted to 100 μ l. The RT efficiency and the absence of genomic DNA in the cDNA were checked by PCR with specific *EF1* α primers surrounding an intron. All primers used for RT-PCR are listed in Table S4.

PCR amplification of genomic DNA and cDNA

Specific PCR primers were designed using the PRIMER3 software available online (Rozen and Skaletsky 2000). Desalted oligonucleotides were synthesized by Sigma-Genosys (Saint Louis, USA). PCR reactions were carried out in 15 μ l with GoTaq[®] flexi DNA Polymerase according to the manufacturer's recommendations (Promega). The general amplification conditions were as follows: 94°C, 2 min; 35 × [94°C, 30 s; annealing temperature, 30 s; 72°C, 1 min]; 72°C, 10 min; 10°C, 10 min. Amplifications were performed in a DNA thermal cycler (PTC-200-MJ Research, Biorad).

Marker development and genetic mapping

Mapping was performed on the HW population (Hibrand-Saint Oyant et al. 2008). Version 4.0 of JoinMap[®] (Van Ooijen, J.W., Kyazma B.V., Wageningen, Netherlands) was used for construction of the integrated genetic map. In a first step, the parental maps were determined using LOD 5.0 and the Kosambi function. The mapping parameters were independence LOD, regression mapping as mapping algorithm and in regression mapping a Jump of 5.0, linkages with a recombination frequency of less than 0.3 and a LOD of more than 2.0, and no third round was performed. The integrated map was built with the join function (combined groups for map integration) using homologous parental LGs.

Polymorphisms were sought between the parents in the mapping population to develop PCR-based markers such as SSCP, CAPS and dCAPS. For SSCP analysis, 6 µl of PCR product was mixed with 4 µl of formamide dye [98% deionized formamide (Sigma-Aldrich), 10 mM ethylenediamine tetra-acetic acid (pH 8.0), 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue], denatured for 5 min at 95°C and quickly cooled on ice. Products were separated 8-12% non-denaturing polyacrylamide on gel (acrylamide:bisacrylamide = $37.5:1, 0.5 \times \text{TBE}$) by electrophoresis at 40 W for 4–6 h in $0.5 \times$ TBE. Gels were pre-run for 30 min under the same electrophoretic conditions, and the improved procedure described by Creste et al. (2001) was used for silver staining of gels. The SSCP markers are listed in Table S2.

For CAPS and dCAPS, restriction enzymes that could generate polymorphisms were identified using online software (http://genoweb.univ-rennes1.fr/Serveur-GPO/ outils_acces.php3?id_syndic=2 or dCAPS Finder 2.0: http://helix.wustl.edu/dcaps/dcaps.html (Neff et al. 1998). Five microliter of PCR product was digested with the appropriate restriction enzyme in a 10 μ l final volume according to the manufacturer's recommendations (Promega). Enzymes and PCR conditions for CAPS and dCAPS are listed in Table S3.

Quantitative real-time PCR analysis

Primers were designed with Beacon Designer 7 (PREMIER Biosoft International) and listed in Table S5. Real-time PCR reactions were performed in triplicate using 3 μ l of RT product (1/50 dilution) in a final volume of 15 μ l containing 1 × IQ SYBR Green Supermix (Bio-RAD), and 0.3 μ M of each primer. Amplifications were performed using an Opticon 4 RealTime PCR detector (Bio-RAD) as follows: 95°C, 3 min; 40 × [95°C, 15 s; 60°C, 1 min]. The amplification specificity was verified by a final dissociation curve ranging from 60 to 95°C. Amplification and dissociation curves were monitored and analysed with Opticon Monitor (Bio-RAD). The amount of plant RNA in each sample was normalized using the *TCTP* gene as reference after checking the homogeneity of the Ct variation with a second housekeeping gene, i.e. *EF1* α . The relative expression level calculation was done according to Pfaffl (2001). The standard error was calculated from three repetitions per sample. Q-PCR experiments were performed according to the recently published recommendations (Gutierrez et al. 2008; Udvardi et al. 2008).

Sequence data from this article have been deposited with the EMBL data library under accession numbers (in parentheses) *RoEMF1* (*FM999793*), *RoEMF2* (*FM999794*), *RoLHP1* (*FM999795*), *RoTFL1* (*FM999796*), *RoGA20OX* (*FM999797*), *RoGA3OX* (*FM999798*), *RoGID1* (*FM999799*), *RoSPINDLY* (*FM999800*), *RoFT* (*FM999826*), *RoLFY* (*FM999801*), *RoAP1a* (*FM999802*) and *RoAP1b* (*FM999803*).

Results

Isolation of flowering-related genes in rose

Based on our knowledge on rose, we mainly focused on isolating genes involved in the GA pathway and floral repressors in *Arabidopsis*. Some of these genes (such as *RoDELLA*) were present in the rose EST database and previously described by Foucher et al. (2008). The other genes were isolated using a degenerate primer strategy. Degenerate primers were designed from conserved sequence blocks. Rose sequences obtained with these degenerate primers

Table 1 Floral genes isolated in rose using degenerate primers

were subjected to tBLASTx searches in turn against *Arabidopsis* nucleic-acid database (TAIR). BLAST hits were visually assessed for degree of amino-acid conservation in order to identify relationships between *Arabidopsis* and rose sequences (Table 1). Phylogenetic analyses were performed for genes belonging to multigenic families.

Floral integrators

MADS-box gene family MADS-box genes encode a family of transcription factors that control a diverse range of processes in flowering plants (Becker and Theissen 2003). MADS-box proteins, represented by more than 100 members in Arabidopsis, are characterized by a highly conserved N-terminal domain. Among these proteins, 39 are sub-classified in the MIKC class (Parenicova et al. 2003). They control flowering time (FLC, SOC1, SVP), floral meristem identity (AP1, FUL, CAL), floral organ identity (AP3, PI, SEP), fruit formation and ovule identity (AG). We identified RoAP1a and RoAP1b genes. The predicted proteins from *RoAP1a* and *RoAP1b* sequences, respectively, showed 77 and 58% identity with the Arabidopsis AP1 gene (Table 1). By phylogenetic analysis, the two genes were found in the SQUAmosa clade (Fig. 1), as defined by Becker and Theissen (2003). This clade contains Arabidopsis AP1, FUL and CAL proteins.

The *FLC* gene was sought for rose in the rose EST database or by the degenerate primer strategy, but no sequence presenting significant similarities with *FLC* was retrieved.

FT/TFL1 gene family FT and *TFL1* are members of the six gene PEBP family in *Arabidopsis* (Kobayashi et al.

Arabidopsis thaliana			Rosa wichurana			Gene family	
Pathway	Gene name TAIR Ref.		Name	Accession nb.	% identity		
Repressors	EMF1	AT5G11530	RoEMF1 (44%)	FM999793	27	Monogenic	
and vernalization	EMF2	AT5G51230	RoEMF2 (45%)	FM999794	45	Monogenic	
	LHP1	AT5G17690	RoLHP1 (73%)	FM999795	42	Monogenic	
	TFL1	AT5G03840	RoTFL1 (74%)	FM999796	77	Multigenic (PEBP)	
Gibberellic acid	GA20OX	AT5G07200	RoGA200X (85%)	FM999797	64	Multigenic	
	GA3OX	AT1G15550	RoGA3OX (67%)	FM999798	60	(Gibberellin oxidase)	
	GID1	AT3G05120	RoGID1 (100%)	FM999799	80	Monogenic	
	SPINDLY	AT3G11540	RoSPINDLY (100%)	FM999800	79	Monogenic	
Floral integrators	FT	AT1G65480	RoFT (97%)	FM999826	74	Multigenic (PEBP)	
and meristem	LFY	AT5G61850	RoLFY (32%)	FM999801	82	Monogenic	
identity genes	API	AT1G69120	<i>RoAP1a</i> (62%)	FM999802	77	Multigenic (MADS box)	
	API	AT1G69120	<i>RoAP1b</i> (70%)	FM999803	58		

Arabidopsis genes are classified according to the different pathways. The gene references are from TAIR (http://www.arabidopsis.org). For the new isolated rose genes, the percentage in brackets represents the percentage of the coding sequence isolated according to the coding sequence in *Arabidopsis*. The percentage identity was determined with the corresponding *Arabidopsis* genes



Fig. 1 Phylogenetic tree of different MIKC/MADS-box genes in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded* area. Sequence accession numbers are: *FUL (AT5G60910), CAL (AT1G26310), AP1* (*AT1G69120), SEP1 (AT5G15800), SEP2 (AT3G02310), SEP3*

1999). Scant knowledge is available about the four other genes, but they may contribute to flowering time regulation (Mimida et al. 2001; Yamaguchi et al. 2005; Yoo et al. 2004). Using degenerate primers, two genes presenting similarity with *FT/TFL1* genes were isolated. One sequence, named *RoFT*, showed 74% similarity with the *FT* gene. The second sequence, i.e. *RoTFL1*, presented 77% similarity with the *TFL1* gene (Table 1). By phylogenetic analysis, *RoFT* appeared in the *FT/TSF* clade, whereas *RoTFL1* branched with *TFL1* and *ATC* (Fig. 2).

Another floral integrator: LEAFY A partial sequence presenting high similarity (82%) with LEAFY from Arabidopsis was isolated (Table 1). The partial region isolated coded for six of the seven helix folds necessary for DNA binding as a dimer of the LEAFY protein (Hamès et al. 2008).

GA metabolism and signalling

GA promotes flowering in *Arabidopsis* under SD (Wilson et al. 1992). GA20OX and GA30X are responsible for the synthesis of active GA (as GA4), whereas GA2OX is involved in the degradation of active GA (Hedden and

(AT1G24260), SEP4 (AT2G03710), FLC (AT5G10140), AGL12 (AT1G71692), AG (AT4G18960), AGL17 (AT2G22630), AGL42 (AT5G62165), SOC1 (AT2G45660), AP3 (AT3G54340), AGL24 (AT4G24540), SVP (AT2G22540), RoSOC1 (CF349866.1), RoAP1a (FM999802), RoAP1b (FM999803), MASAKOC1/RAG (AB025644), MASAKO BP (AB038462), MASAKO B3 (AB055966), MASAKOeuB3 (AB099875)



Fig. 2 Phylogenetic tree of FT/TFL1 gene family in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded area*. Sequence accession numbers are: *FT* (*AT1G65480*), *TSF* (*AT4G20370*), *MFT* (*AT1G18100*), *BFT* (*AT5G62040*), *ATC* (*AT2G27550*), *TFL1* (*AT5G03840*), *RoFT* (*FM999826*), *RoTFL1*(*FM999796*)



Fig. 3 Phylogenetic analysis of GA oxidase genes in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded area*. Sequence accession numbers are: *GA200X1* (*AT4G25420*), *GA200X2* (*AT5G51810*), *GA200X3* (*AT507200*), *GA200X4* (*AT1G60980*), *GA200X5*

(AT1G44090), GA3OX1 (AT1G15550), GA3OX2 (AT1G80340), GA3OX3 (AT4G21690), GA3OX4 (AT1G80330), GA2OX1 (AT1G78440), GA2OX2 (AT1G30040), GA2OX3 (AT2G34555), GA2OX4 (AT1G47990), GA2OX6 (AT1G02400), GA2OX7 (AT1G50960), GA2OX8 (AT4G21200), RoGA2OOX (FM999797), RoGA3OX (FM999798), RoGA2OX (BQ105545.1)

Phillips 2000). These genes are encoded by a multigenic family and have 20-Fe-oxydoreductase activity (Hedden and Phillips 2000). We identified RoGA20OX and RoGA3OX that presented more than 60% identity with AtGA200X and AtGA30X, respectively (Table 1). Phylogenetic analysis revealed that the three rose genes belonged to the respective Arabidopsis clades (Fig. 3). Moreover, the specific domains described by Sakamoto et al. (2004) were found in rose sequences. RoGA20OX had the NYYPXCXXP and LPWKET domains as well as AtGA20OX. Moreover, His93, His 252 and 308 were conserved in RoGA20OX. Asp242, His248 and His296, which allow cofactor interaction, were present in RoGA3OX. And finally, RoGA2OX was found to have His217, H278 and Asp227, which are supposed to fix Fe++.

Concerning GA signalling, we isolated genes presenting similarities with *GID1* and *SPINDLY* (Table 1). RoGID1 shares 80% identity with the *Arabidopsis* GA receptor GID1 (Nakajima et al. 2006) and presents the two amino acids (Gly196 and Arg251) that are essential for in vitro interaction between GID1 and GA4 (Nakajima et al. 2006). We identified one sequence encoding an O-linked *N*-acetylglucosamine

transferase (OGT), i.e. RoSPY, which shared 79% identity with AtSPY (Table 1). SPY contains multiple copies of N-terminal tetratricopeptide repeats (TPR) and two conserved domains (CDI and CDII) in C-termini common to animal OGTs (Silverstone et al. 2007). *RoDELLA*, one homologue of *AtRGA*, and *RoSLEEPY*, a homologue of AtSLY, were previously described by Foucher et al. (2008). Full-length cDNAs were isolated for the four GA signalling genes.

Isolation of floral repressors in rose

In addition to the floral repressor *RoTFL1*, three other sequences presenting similarities with *Arabidopsis* floral repressors were isolated in rose: *RoEMF1*, *RoEMF2* and *RoLHP1*. The *RoEMF1* gene only showed similarity with *EMF1* genes previously isolated in rice and *Arabidopsis* (Table 1, Aubert et al. 2001). The *RoEMF1* partial sequence is predicted to encode the LXXLL motif that is thought to mediate steroid receptor binding. This motif is also found in rice and *Arabidopsis* (Aubert et al. 2001). As previously described for *EMF2* in *Arabidopsis* (Yoshida et al. 2001), the predicted protein for *RoEMF2* contained a

Table 2	Genetic markers, polymorphisms in the HW population and	ł
location	of rose flowering genes on the genetic map	

Name	MM	Polymorphism	
RoEMF1	dCAPS	Male	7
RoEMF2	SSCP	Male	5
RoELF8	CAPS	Female	2
RoVIP3	dCAPS	Male	4
RoLHP1	SSCP	Male	1
RoTFL1	dCAPS	Male	1
RoGA20OX	SSCP	Female	1
RoGA3OX	SSCP	Male	2
RoGA2OX	SSCP	Male	6
RoGID1	SSCP	Male	4
RoDELLA	SSCP	Male	4
RoSPINDLY	SSCP	Male	4
RoSLEEPY	SSCP	Male	7
RoGI	SSCP	Male	7
RoCOL1	_	Monomorphic	-
RoCOL2	SSCP	Female	3
RoFT	SSCP	Male/female	3
RoSOC1	CAPS	Male	1
RoLFY	SSCP	Female/male	5
RoAP1a	SSCP	Female/male	5
RoAP1b	SSCP	Female	2
MASAKOB3	SSCP	Female	2
MASAKOeuB3	SSCP	Male	7
MASAKOBP	CAPS	Male	7
MASAKOC1/RAG	dCAPS	Female/male	6

MM molecular markers, LG linkage group

single C2H2 zinc finger domain and an acidic W/M domain in the C-term part. LHP1 in *Arabidopsis* showed a structure similar to HETEROCHROMATIN PROTEIN 1 (HP1) from *Drosophila* with the two characteristic HP1 motifs, the chromo domain and the chromo shadow domain (Gaudin et al. 2001). Both domains were found in the predicted protein encoded by *RoLHP1*.

Using degenerate primers (Table 1), we were able to isolate 12 new genes with a potential role in flowering control. Combined with a previous approach based on rose EST database screening (Foucher et al. 2008), we now have a set of 25 genes (Table 2) presenting significant similarities to genes involved in floral initiation and development. To further characterize these genes, we searched for co-localization with previously described loci involved in flowering in rose.

Genetic mapping

Markers (SSCP, CAPS or dCAPS) were developed for mapping candidate genes (Table 2), and were used to expand genetic maps of the interspecific F1 progeny (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008). Of the 25 candidate genes analysed, 24 were assigned on a genetic map at a LOD score of 5.0. RoCOL1 was monomorphic and could not be mapped. An integrated map consisting of seven linkage groups with 213 markers spanning 482 cM was built (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008; this work). Candidate genes were distributed throughout the seven linkage groups (Fig. 4). Three of the new mapped genes (RoFT, RoAP1b and RoGA3OX) departed from the expected Mendelian segregation (P < 0.05). Three genes involved in GA signalling (*RoSPY*, RoDELLA and RoGID1) and one involved in the vernalization response (RoVIP3) were located on linkage group 4, where the RECURRENT BLOOMING locus and a QTL for blooming date were previously localized (Hibrand-Saint Oyant et al. 2008). In the 91 individuals of the mapping population, no recombinant was found between RB and RoSPY. Furthermore, RoSPY, RoDELLA and RoVIP3 were located within the confidence interval of the OTL for blooming date.

Where are flowering-related genes expressed in rose?

The presence of transcripts was tested by reverse-transcription PCR in different tissues for all genes studied (Fig. 5). The experiment was performed on seven Rosa wichurana tissues: roots, leaves, shoots, floral buds and three types of growing apices (vegetative apices, vegetative pre-floral apices and floral apices). Nine genes (RoEMF2, RoGA200X, RoGA3OX, RoGA2OX, RoGID1, RoSPY, RoSLY, RoSOC1, *RoCOL1*) were expressed in all tested tissues (Fig. 5). *RoGI* and RoDELLA were expressed everywhere except in floral buds, whereas RoEMF1, RoVIP3 and RoLHP1 were expressed everywhere except in leaves. Few genes showed more specific expression patterns. RoELF8 transcripts were detected in apices, shoots and roots. RoCOL2 was expressed in apices, leaves and floral buds. RoTFL1 was mainly expressed in roots and vegetative apices. Few genes were expressed only during the floral process. RoLFY and RoAP1b transcripts were present in induced and floralinduced apices as well as floral buds. RoFT transcripts were accumulated in floral apices and floral buds. Finally, the expression of MASAKO genes and RoAP1a was tissue specific: MASAKO genes were only expressed in floral buds and RoAP1a was only expressed in floral apices.

Analysis of transcript abundance

To examine whether the floral phenotype could be related to floral gene expression, transcript levels of nine previously isolated flowering-related genes were analysed by qRT-PCR in a couple of mutants diverging by the recurrent



Fig. 4 Integrated genetic map of the HW population. Flowering genes are indicated in bold, SSR markers in normal type. To facilitate reading, AFLP markers are not presented in the figure. Map distances (in cM) are listed on the *left* and loci on the *right* of each linkage group. Distorted markers are indicated with an *asterisk* *P < 0.001. Additive

QTLs for blooming date (BD) and NP are represented by a box prolonged with lines, which, respectively, span the LOD1 and LOD2 confidence intervals (as previously described by Hibrand-Saint Oyant et al. (2008)

flowering trait. Cv Félicité&Perpétue (FP) is a non-recurrent rose flowering only in spring, whereas its vegetative mutant, cv Little White Pet (LWP) is recurrent and flowers during all favourable seasons. Floral initiation was determined by dissection and observation of the meristem under a binocular microscope. We considered that floral initiation happens before the first morphological changes associated with floral development were observed in the meristem (data not shown). For both cultivars, we considered that floral initiation took place before 5 April (in Fig. 6, floral tissues are indicated by grey boxes). Then, kinetics represent the first floral initiation for LWP and the unique floral initiation for FP in the year. Transcripts were detected in all 144 measurements, showing that the investigated genes were expressed during floral initiation in spring and autumn for both cultivars. Only one gene, i.e. RoSPY, showed no variation in transcript abundance among the tested samples. The transcript accumulation of RoFT, RoLFY, RoAP1b and RoGID1 increased during spring and seemed to be correlated with the flowering time of LWP and/or FP. RoFT induction occurred in two waves: the first started on 6 March and the second on 12 April, resulting in an accumulation of 15-fold and 57-fold more transcripts for FP and LWP, respectively. In autumn (1 October), the transcript abundance was comparable to the level in spring before floral initiation. The same pattern was observed for *RoLFY* and RoAP1b. For the three genes, the level was low in autumn in vegetative buds. RoGID1 was also induced in LWP in spring, whereas it remained stable in FP. For RoD-ELLA and RoSLY, the transcript level was quite stable for FP. A weak transient increase was detected in spring for LWP ($\times 2$ on 13 March for *RoDELLA* and $\times 2.5$ on 23 March for RoSLY). The gene encoding one enzyme of active GA synthesis, i.e. RoGA200X, was repressed (4 times) until 13 March, and then increased to 0.6 and 0.7, respectively, on 23 March and 5 April, and then decreased again. In LWP, after a decrease, transient overexpression was detected on 23 March (\times 4), then the transcript decreased as rapidly as for FP. RoGA2OX, encoding a GA inactivation enzyme, showed a complex expression profile. In FP, transcripts accumulated strongly on 6 March, then decreased on 13 March to progressively increase later in spring. In LWP RoGA2OX, the transcript abundance was high until 6 March, and then fell on 13 March, and increased again during spring. In October, the RoGA2OX transcript abundance was equal to the level of 13 March in FP and LWP.

Discussion

Flowering genes are conserved in rose

Except for *FLC*, all genes we looked for in rose were found among the four different floral pathways, floral integrators and organ identity genes. The mean similarity between rose and *Arabidopsis* sequences was as high as 65% and reached 82% for *RoLFY*, for example. For single genes such as *SPY*, *RoSPY* showed high similarity with the *Arabidopsis* sequence and presented conserved domains. We are thus


Fig. 5 RT-PCR analysis of rose gene expression. Specific primers for each gene were used to amplify the cDNA. PCR was performed on cDNA obtained from different tissues: leaves (L), apices from indoor grown roses (A), internodes (I), roots (R), apices during floral transition (Av, before the first floral modification; Af, after the first morphological changes) and floral buds (Fb)

confident that *RoSPY* is the homologue of *SPY*. The same conclusions are drawn for *RoLFY*, *RoLHP1*, *RoEMF2* and *RoGID1*, which were found to be similar to *LFY*, *LHP1*, *EMF2* and *GID1*, respectively. For *EMF1*, the percentage similarity was low [27% between *RoEMF1* and *EMF1* (Table 1)]. This value is in the same range as the similarity between *Arabidopsis* and rice sequences (31% between *SPY* in *Arabidopsis* and its homologue in rice). Furthermore, *RoEMF1* showed a conserved domain characteristic of *EMF1* (Aubert et al. 2001). Therefore, we propose that *RoEMF1* is the homologue of *EMF1*.

We also performed a phylogenetic analysis for genes belonging to multigenic families such as MADS-box genes. The two MADS-box genes isolated in rose clearly belonged to the AP1 clade (SQUAMOSA; Fig. 1; Becker and Theissen 2003). We were unable to isolate a gene with similarity to *FLC*, which is a major floral repressor in *Arabidopsis* (Michaels and Amasino 1999). *FLC* exists in other **Fig. 6** Expression pattern of nine flowering genes during the floral process determined by qRT-PCR. **a** floral integrators, **b** GA metabolism, **c** GA signalling. The X-axis indicates the dates at which apices were sampled in 2007. Data are ratios of expression according to Pfaffl (2001) ±SE (for three replicates). For each gene, transcript levels are expressed relative to the first sample of FP (16 February, base value = 1). Apices were harvested from new shoots grown from budbreak to autumn in the non-recurrent cv Félicité&Perpétue (*black bar*) and the recurrent cv Little White Pet (*grey bar*). Floral initiation was determined according to Foucher et al. (2008) by binocular observation. Floral tissues (after floral initiation) are represented in a *grey box* (sample since 5 April)

Brassicaceae such as Brassica oleracea (Okazaki et al. 2007), but no FLC has been detected in rice (Izawa et al. 2003). Recently, Reeves et al. (2007) revealed two FLC homologues in sugar beet and assumed that FLC homologues likely exist in other Eudicot lineages. Moreover, sequence analysis of the grapevine genome shows two sequence homologues of FLC as well (Diaz-Riquelme et al. 2009). Therefore, we cannot exclude the possibility that the absence of a rose FLC in our study could be explained by a failure to amplify the rose homologue with degenerate primers. Phylogenetic analysis of FT/TFL1 (Fig. 2) and GA oxidase (Fig. 3) confirmed the similarity between rose and Arabidopsis genes. Based on sequence similarities and conserved domains, we conclude that we have isolated rose genes that are putative homologues to genes in Arabidopsis thaliana.

However, in our study, for most of the genes, we only managed to isolate one homologue per gene and other paralogues may exist. As paralogues can act in specific processes, a role of the isolated genes in flowering cannot be ascertained. For example, there are five DELLA proteins in *Arabidopsis* that play redundant and specific roles in different processes: RGL2, RGA and RGL1 in flower development (Cheng et al. 2004; Tyler et al. 2004; Yu et al. 2004), RGA and GAI in stem elongation and root growth (Fu and Harberd 2003), RGL2, RGL1 in seed germination (Cao et al. 2006; Lee et al. 2002). In rose, we have only isolated one gene, i.e. *RoDELLA*, but additional DELLA proteins may occur as previously shown in apple, where six DEL-LAs were isolated by Foster et al. (2007).

We performed expression analyses to further study the homologue genes in rose and tried to obtain evidences of their involvement in the floral process. RT-PCR experiments (Fig. 5) showed that each gene was expressed during the floral process. Moreover, some genes such as organ identity genes (*MASAKO* genes) were only expressed in floral tissues, as previously shown (Hibino et al. 2006; Kitahara et al. 2001; Kitahara and Matsumoto 2000) (Fig. 5). Furthermore, *RoFT*, *RoLFY* and *RoAP1* are induced during the floral process, as previously shown in *Arabidopsis* (Lee et al. 2006; Wigge et al. 2005) (Fig. 6c).



They could therefore be useful as markers of floral initiation in rose. Key floral genes (floral integrators, floral identity genes and organ identity genes) seemed to be expressed in a similar way as in *Arabidopsis*, thus suggesting that the floral gene network might be conserved in rose. The same conclusions were drawn in pea (Hecht et al. 2005), grapevine (Carmona et al. 2007) and rice (Izawa 2007).

In summary, by combining the EST search (Foucher et al. 2008) and the degenerate primer strategy (this study), we studied 26 genes putatively homologous to genes involved in floral initiation and development in *Arabidopsis*.

Genetic linkage between floral traits and floral genes

To further investigate the role of these genes in flowering control processes in rose (floral initiation as well as recurrent blooming), we looked for co-localization with loci controlling flowering in rose and the analysed transcript abundance of candidate genes in a non-recurrent rose and in its spontaneous vegetative recurrent mutant.

Interestingly, three genes, i.e. RoVIP3, RoSPY and RoD-ELLA, were found in the vicinity of two important loci: a QTL governing the flowering date (or precocity) and the RECURRENT BLOOMING locus. No recombination was found between RB and RoSPY when 91 individuals were analysed. These genetic data indicate a possible role of GA signalling in flowering control in rose (flowering precocity and recurrent blooming). Roberts et al. (1999) already proposed that GA may be involved in flowering in rose. Exogenous application of GA inhibits flowering in non-recurrent roses, whereas it has no effect on flowering in recurrent roses (Roberts et al. 1999). As RoVIP3, a homologue of VIP3 (VERNALIZATION INDEPENDENCE 3), is also a putative candidate for the QTL of precocity, we cannot exclude a role of vernalization in the control of flowering in rose. Indeed, recurrent roses are self inductive, whereas vernalization is necessary for flowering in non-recurrent roses (Foucher F., personal communication). Therefore, we hypothesise that the recurrent blooming mutation could affect GA signalling as well as the vernalization response. In Lolium temulentum, a perennial plant, vernalization affects GA signalling, i.e. without vernalization, plants are unable to respond to GA (McMillan et al. 2005). After vernalization, a long-day treatment is necessary for GA synthesis, which in turn induces flowering (King et al. 2003).

Gibberellins control rose flowering

To further explore the role of GA in flowering in rose, we analysed the expression of GA genes isolated in rose during the floral process in a pair of rose mutants for recurrent blooming, i.e. FP and LWP.

GA metabolism and floral initiation

In early March, before morphological changes, we observed a peak of RoGA2OX expression in both FP and LWP (Fig. 6b) that might be responsible for greater GA degradation activity, whereas RoGA20OX was repressed (no GA synthesis). Indeed, a correlation between the GA metabolism gene transcript level and the active GA content has been reported in other plants (Oh et al. 2006; Yamaguchi et al. 1998). Repression of RoGA200X and induction of RoGA2OX might cause a decrease in GA content. In the same genotypes (FP and LWP), low GA content was already observed in early March (Roberts et al. 1999). Therefore, we proposed that RoGA2OX induction and RoGA20ox repression lead to low GA content, which enable floral initiation to occur in early spring in recurrent and non-recurrent roses. It would be interesting to determine what signals control GA degradation in rose (link with vernalization/photoperiod). Photoperiodic control of GA metabolism could thus be an interesting focus of further studies. Indeed, in Lolium perenne, LD induces GA metabolism genes and then induces flowering in GA responding plants (King et al. 2006; McMillan et al. 2005).

GA signalling and recurrent blooming

We detected co-localization between a GA signalling gene, RoSPY, and the RECURRENT BLOOMING locus (RB, Fig. 4). Furthermore, a gene potentially involved in the early steps of GA signalling (RoGID1) showed different expression patterns when FP and LWP (recurrent blooming mutation) were compared. In FP, GA signalling genes were expressed at the same level during the floral process, whereas in the mutant (LWP), RoGID1 was upregulated after the floral initiation (Fig. 6c). The present analysis was based on only 91 individuals: further studies using a larger sample are needed for a closer investigation of the linkage between RB and RoSPY. Moreover, Roberts et al. (1999) showed that an exogenous application of GA has no effect on flowering in recurrent roses whereas it inhibits flowering in non-recurrent roses. From these different results, we hypothesise that RB mutation could affect GA signalling and RoSPY could be a candidate for GA signalling disruption. Nevertheless, RoSPY remained stable at the transcriptional level in both recurrent and non-recurrent rose. Further experiments are therefore needed to clarify the link between RoSPY and the recurrent blooming phenotype.

To conclude, the flowering gene network seems to be conserved in rose. We isolated and characterized genes involved in each of the four floral pathways, floral integrators, meristem identity genes and organ identity genes. The GA pathway appears to be a key regulator of flowering in rose; GA metabolism might be responsible for floral initiation and GA signalling might be involved in recurrent blooming control.

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DETERMINATE and LATE FLOWERING Are Two TERMINAL FLOWER1/CENTRORADIALIS Homologs That Control Two Distinct Phases of Flowering Initiation and Development in Pea

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Genes in the *TERMINAL FLOWER1* (*TFL1*)/*CENTRORADIALIS* family are important key regulatory genes involved in the control of flowering time and floral architecture in several different plant species. To understand the functions of *TFL1* homologs in pea, we isolated three *TFL1* homologs, which we have designated *PsTFL1a*, *PsTFL1b*, and *PsTFL1c*. By genetic mapping and sequencing of mutant alleles, we demonstrate that *PsTFL1a* corresponds to the *DETERMINATE* (*DET*) gene and *PsTFL1c* corresponds to the *LATE FLOWERING* (*LF*) gene. *DET* acts to maintain the indeterminacy of the apical meristem during flowering, and consistent with this role, *DET* expression is limited to the shoot apex after floral initiation. *LF* delays the induction of flowering by lengthening the vegetative phase, and allelic variation at the *LF* locus is an important component of natural variation for flowering time in pea. The most severe class of alleles flowers early and carries either a deletion of the entire *PsTFL1c* gene or an amino acid substitution. Other natural and induced alleles for *LF*, with an intermediate flowering time phenotype, present no changes in the PsTFL1c amino acid sequence but affect *LF* transcript level in the shoot apex: low *LF* transcript levels are correlated with early flowering, and high *LF* transcript levels are correlated with late flowering. Thus, different *TFL1* homologs control two distinct aspects of plant development in pea, whereas a single gene, *TFL1*, performs both functions in Arabidopsis. These results show that different species have evolved different strategies to control key developmental transitions and also that the genetic basis for natural variation in flowering time may differ among plant species.

INTRODUCTION

For some fundamental aspects of plant biology, the genes involved have been identified through a molecular genetics approach using the model species Arabidopsis. From this basic information, comparative studies between species can begin, in particular to understand the genetic and molecular mechanisms responsible for the large diversity in plant morphology and to identify the genes involved in adaptive evolution (Cronk, 2001). In plants, the best example of such evolutionary developmental studies is the identification and analysis of MADS box genes involved in flower development in several plant species, including gymnosperms (reviewed by Ma and De Pamphilis, 2000). Isolation of putative orthologs in different species and studies of RNA and/or protein expression patterns provide insights into the conservation and diversification of gene function in plant development (Hofer and Ellis, 2002). For instance, the *LEAFY* (*LFY*) gene of Arabidopsis, which was isolated initially as *FLORICAULA* in snapdragon, is a key gene involved in floral development (Coen et al., 1990; Weigel et al., 1992). Orthologs of *LFY* have since been studied in numerous other species, including *UNIFOLIATA* in pea (Hofer et al., 1997). In certain cases, different regulator processes or new roles can be found. For example, *UNIFOLIATA* was proposed to regulate indeterminacy during both leaf and flower development. The function of *LFY* during leaf development was not described (Hofer et al., 1997).

Flowering time is a major adaptive trait in the life strategy of flowering plants, which have to synchronize their reproduction with favorable environmental conditions. After a vegetative phase, plants undergo the floral transition. The switch from the vegetative to the reproductive stage is controlled by physiological signals and genetic networks that integrate environmental (photoperiod and temperature) and endogenous (stage of the plant) conditions (Levy and Dean, 1998; Colasanti and Sundaresan, 2000). The molecular genetics of the long-day plant Arabidopsis enable the isolation and characterization of the genes that control flowering time (reviewed by Mouradov et al., 2002). In agronomic species, most of the genetic loci that control flowering time have been identified as quantitative trait loci (in maize or

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rice; Yano et al., 2001; Salvi et al., 2002) or as mutants (in pea; Murfet and Reid, 1993). Using a combination of map-based cloning and a candidate-gene approach, two quantitative trait loci, *Hd1* and *Hd3a*, have been cloned in rice (Yano et al., 2000; Kojima et al., 2002) and one, *VRN1*, has been cloned in wheat (Yan et al., 2003). They correspond to genes that are similar to the Arabidopsis genes *CONSTANS*, *FLOWERING LOCUS T (FT)*, and *APETALA1*, respectively.

Because pea is both a classic model species for plant development and an important crop in Europe, we used a molecular approach to study homologs of the snapdragon CENTRORA-DIALIS/Arabidopsis TERMINAL FLOWER1 (CEN/TFL1) genes in pea. CEN is involved in inflorescence architecture in snapdragon (Bradley et al., 1996). The cen mutation leads to the conversion of the indeterminate inflorescence to a terminal flower. Orthologs of CEN have been found in different species: TFL1 in Arabidopsis (Bradley et al., 1997), SELF PRUNING (SP) in tomato (Pnueli et al., 1998), CET in tobacco (Amaya et al., 1999), and LpTFL1 in Lolium perenne (Jensen et al., 2001). In Arabidopsis, tfl1 mutants have a terminal flower and flower earlier than the wild type (Bradley et al., 1997). This early-flowering phenotype was not observed in snapdragon. TFL1 may play a role in inflorescence meristem identity as well as in floral initiation control as a repressor of flowering. It was proposed that these two distinct roles are in fact one, with TFL1 controlling the length of both the vegetative and reproductive phases (Ratcliffe et al., 1998).

CEN and TFL1 are similar to a family of mammalian phosphatidylethanolamine binding proteins (PEBPs) also known as Raf-1 kinase inhibitor proteins. Crystallography analysis reveals that CEN may be involved in interaction with a kinase (Banfield and Brady, 2000). In tomato, SP was shown to interact with multiple proteins and was proposed to encode a modular protein with the potential to interact with a variety of signaling pathways (Pnueli et al., 2001). Expression analysis has revealed that genes closely related to TFL1 are expressed mainly in the shoot apical meristem in the region below the terminal meristem. CEN is induced during floral initiation (Bradley et al., 1996), whereas TFL1 expression also is found during the vegetative phase; this expression could explain the role of TFL1 in delaying flowering in Arabidopsis (Bradley et al., 1997). Analysis of mutants and sequencing of the entire Arabidopsis genome have revealed that the TFL1 genes belong to a small family (at least six genes) with functional divergence (Mimida et al., 2001). One of them, FT, has a TFL1-antagonistic role by promoting flowering in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999). Studies of TFL1 homologs in other species may help us better understand the function and the evolution of the TFL1 family in flowering plants.

Floral initiation and development in pea have been studied for many decades (Murfet and Reid, 1993). Based on physiological and mutational analyses, a model for flowering that involves both a floral inhibitor and a stimulus has been developed (reviewed by Reid et al., 1996; Weller et al., 1997). The stimulus is specific to flowering and is under the control of *GIGAS* (Beveridge and Murfet, 1996). The synthesis of the floral inhibitor is controlled by different genes (*STERILE NODE*, *HIGH RE-SPONSE*, *PHOTOPERIOD*, *DAY NEUTRAL*, and *EARLY*) and is strongly regulated by photoperiod. The integration of the signals occurs in the apex and is controlled by the *LATE FLOW-ERING (LF)* gene. *LF* determines the node of flowering in pea for a given genetic background (Figure 1). Four natural and induced classes of alleles are known—*Lf-d*, *Lf*, *If*, and *If-a*—that in maximal inductive conditions confer minimum nodes of flowering of 15, 11, 8, and 5, respectively. The dominance order is *Lf-d* > *Lf* > *If* > *If-a*, with the *If-a* allele being recessive (Murfet, 1975). *LF* is active in the shoot, and the different alleles determine the threshold of sensitivity of the apical meristem to flowering signals. Because mutations of *LF* lead to plants with an early phenotype, *LF* may be considered a repressor of flowering.

Pea is an indeterminate-flowering plant, as is Arabidopsis. After floral initiation, the shoot apical meristem is converted to an inflorescence meristem (called I1; Figure 1). The I1 meristem grows indefinitely, and an axillary meristem in the leaf axil generates a secondary inflorescence, I2. Flowers (often two) arise laterally from I2. At the onset of senescence, the I2 meristem ceases growing and is converted to a stub, a terminal meristem with epidermal hairs (Singer et al., 1999). A pea mutant, known as determinate (det), produces a few axillary flowers and an apparent terminal flower (Reid and Murfet, 1984; Singer et al., 1990) (Figure 1). Scanning electron microscopy showed that this terminal flower actually arises from an axillary meristem and that the I1 meristem is converted to a stub as in the wild type (Singer et al., 1990) (Figure 1). A real terminal flower can be obtained in pea by crossing the det mutant with another mutant, vegetative1, which remains vegetative (Reid and Murfet, 1984; Singer et al., 1999).

Using degenerate primers, we isolated three TFL1 homologs in pea. Detailed analyses, by gene mapping, allele sequencing, and expression studies, revealed that two of these homologs correspond to important genes involved in flower initiation and development in pea: *DET* and *LF*. This study provides compar-



Figure 1. Scheme of the Phenotypes of If-a, det, and If-a det in Pea.

Black arrows represent shoot apical meristems, open circles represent flowers, and closed circles represent stubs (terminal meristems with epidermal hairs). I1 and I2 indicate the primary and secondary inflorescence meristems, respectively. ative information about the function and evolution of *TFL1* genes in flowering plants.

RESULTS

Arabidopsis *TFL1* Homologs Represent a Small Gene Family in Pea

To isolate TFL1-related sequences in pea, we designed several different degenerate primers corresponding to conserved domains identified from the alignment of published TFL1 homologs (Figure 2A) and other homologous EST sequences. Two different primer pairs successfully amplified fragments from pea genomic DNA. Two fragments of 450 bp (A) and 850 bp (B) obtained with the primer combination TFL1-3/TFL1-5, and a single fragment of 450 bp (C) obtained using the primer combination TFL1-1/TFL1-2, were isolated and sequenced. Each band yielded sequence similar to that of *TFL1* and *CEN*. Additional sequences for each fragment were obtained by 3' and 5' rapid amplification of cDNA ends PCR on seedling or flower cDNA (see Methods). Complete sequences were obtained for transcripts corresponding to fragments A and C, and these genes were designated PsTFL1a and PsTFL1c, respectively. For the third gene, designated PsTFL1b, only a partial sequence was obtained.

To evaluate the number of *TFL1*-related genes in pea, we made a DNA gel blot using *PsTFL1a* as a probe (Figure 3). Three hybridizing bands were seen in HindIII and EcoRV digests, and four bands were seen in digests with EcoRI. The additional band in the EcoRI digest can be explained by the presence of an EcoRI site in the *PsTFL1b* sequence. Therefore, we concluded that the three *TFL1* homologs isolated probably represent the entire *TFL1* family in pea.

PsTFL1a and *PsTFL1c* are predicted to encode proteins of 174 and 173 amino acids, respectively, according to the computer software Eugène (Schiex et al., 2000). The predicted PsTFL1a and PsTFL1c proteins show 70% amino acid identity and 72 and 65% identity with *TFL1*, respectively. The *PsTFL1b* clone, which is incomplete at the 5' end, covers 90 amino acids and shows 73% identity with *TFL1* over this region. The protein sequence alignment in Figure 2A shows that the three predicted pea proteins contain large regions that are conserved across TFL1 homologs from other species. Intron/exon boundaries also are highly conserved across these genes (data not shown).

Previous studies have shown that despite their apparently similar functions, Arabidopsis *TFL1* and *Antirrhinum CEN* are not particularly closely related (Mimida et al., 2001). Clustering of *TFL1*-related sequences based on amino acid similarity suggested the presence of several distinct groups (Figure 2B). Both *PsTFL1a* and *PsTFL1c* cluster with *TFL1*. However, *PsTFL1b* belongs to another group of genes that includes *CEN*, *SP*, and *ATC*. Although *PsTFL1b* is a partial sequence, the same results were obtained when the analysis was performed using only the C-terminal region. Other members of the Arabidopsis *TFL1* family, such as *FT*, *TSF*, and *BFT* (Mimida et al., 2001), are more distant (Figure 2B).



Figure 2. Comparison of Pea TFL1 Homologs with TFL1 Related Genes.

(A) Alignment of the predicted amino acid sequences of *PsTFL1a*, *PsTFL1b*, and *PsTFL1c*, *TFL1* (Bradley et al., 1997), *SP* (Pnueli et al., 1998), *CEN* (Bradley et al., 1996), *ATC* (Mimida et al., 2001), and *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). The alignment was performed with Multialign software (Corpet, 1988). Arrows represent the positions of the degenerate primers used to isolate the *TFL1* homologs in pea.

(B) Phylogenic tree of TFL1-related proteins constructed using the NJ method with the program CLUSTAL W. Branches with a bootstrap value of >600 (of 1000) are shown with thick lines. In addition to the proteins shown in **(A)**, TSF, BFT, MFT, CET1, CET2, and CET4 from tobacco (Amaya et al., 1999) and BnTFL1-1 from *Brassica napus* were included in the analysis.

The Map Locations of *PsTFL1a* and *PsTFL1c* Suggest That They May Be Candidate Genes for *DET* and *LF*, Respectively

To study the relationship between the *TFL1*-like sequences isolated and already known flowering loci in pea, we developed molecular markers corresponding to these sequences. We screened the parents of two different mapping populations for single nucleotide polymorphisms (SNP) and converted these to PCR-based cleaved amplified polymorphic sequence or derived cleaved amplified polymorphic sequence markers (see Methods).

PsTFL1a was mapped in the Térèse \times K586 RIL population (Laucou et al., 1998) and was found to be located in group V between two RAPD markers, K3-3000 and D3-1100, a region shown previously to contain *DET* (Rameau et al., 1998). Analysis of an F2 population segregating for a *det* mutation showed no recombinant genes between *DET* and *PsTFL1a* among 120 individuals (data not shown). Thus, *PsTFL1a* is closely linked to *DET*, and in view of the phenotypic similarity between *det* mutants and Arabidopsis *tfl1* mutants, we considered it to be a good candidate for the *DET* gene.

Because of the lack of SNP for *PsTFL1b* and *PsTFL1c* between Térèse and K586, these genes were mapped in another RIL population derived from a cross between the more distant lines JI 281 and JI 399 (Ellis et al., 1992). *PsTFL1b* maps on linkage group III close to the markers PsZF18 and C7/1 (Ellis et al., 1992; Laucou et al., 1998), a region containing no obvious candidate flowering loci. By contrast, *PsTFL1c* was located in linkage group II and segregated with the B5/9 marker in a re-



Figure 3. DNA Gel Blot of the Wild-Type Pea Lines Térèse and Paloma and the Mutant *det-1* (JI 2121).

Genomic DNA was digested with EcoRV, HindIII, and EcoRI. The blot was hybridized with a probe corresponding to *PsTFL1a*. Hybridization and washing conditions were low stringency (temperature of 60° C, 2× SSC as wash salt). Sizes of the marker fragments are represented in kilobases. Pa, Paloma; Te, Térèse.

gion containing *LF* (Ellis et al., 1992; Laucou et al., 1998) and an important quantitative trait loci for flowering time (our unpublished results). This finding suggests that *PsTFL1c* could be a candidate gene for *LF*. Our subsequent analyses focused on *PsTFL1a* and *PsTFL1c*, because these potentially represented genes that regulate agronomically important flowering traits in pea.

det Mutants at the PsTFL1a Locus Carry Significant Mutations

To examine the relationship between *PsTFL1a* and *DET*, we next sequenced *PsTFL1a* from three different *det* mutants and their corresponding wild-type lines when available (Table 1). All three were found to contain SNP within the *PsTFL1a* gene.

In the *det-1* mutant line JI 2121 (Swiecicki, 1987), a point mutation was found at the first exon-intron junction, converting the consensus splicing donor motif TGGT to TGAT (Table 1). Using primer pairs surrounding the first and the second introns, reverse transcriptase–mediated (RT) PCR performed on root RNA from *det-1* and the Paloma wild-type line confirmed that the first intron is not spliced out in *PsTFL1a* transcripts from *det-1*, whereas the second intron is spliced out normally in both the wild type and *det-1* (Figure 4). Retention of the first intron in *det-1* is predicted to result in the introduction of a stop codon, truncating PsTFL1a at amino acid 72.

The *det-2* line JI 1358 arises as a spontaneous mutant, for which no progenitor line is available (J. Hofer, personal communication). We found five SNP in *PsTFL1a* between the *det-2* line and wild-type cv Térèse, some of which may represent natural polymorphism (Table 1). Of these, three are predicted to direct changes in the protein sequence. Two of these changes (Met-4 to lle and Thr-47 to Pro) affect residues that are not notably conserved across the TFL1 family, whereas the Gln residue substituted for in the third change (Gln-127 to Arg) is invariant across all TFL1 sequences and is conserved even in more distantly related proteins such as FT and animal PEBPs. The high degree of conservation implies that mutation of this Gln-127 likely affects TFL1 function and could account for the *det-2* phenotype.

The *det-3* mutant was obtained by mutation of the SG line (Berdnikov et al., 1999), but this line has since been lost. Thus, as in the case of *det-2*, no progenitor line was available for *det-3*. Sequencing revealed five SNP between *det-3* and wild-type cv Térèse (Table 1). Three of these are predicted to direct changes in the protein sequence. The first is Met-4 to lle, which it shares in common with *det-2*. The second is Thr-66 to lle. This residue is conserved perfectly across all known TFL1 sequences. Furthermore, the corresponding substitution in Arabidopsis TFL1 is the basis for the *tf/1-14* mutant phenotype (Ohshima et al., 1997) and was shown to suppress interactions with putative partners (Pnueli et al., 2001). The third change occurs at position 104, where a conserved Glu is replaced by Lys.

Additional evidence for the importance of the Gln-127 and Thr-66 residues comes from a detailed analysis of the CEN (Banfield and Brady, 2000) and TFL1 (our unpublished data) protein structures. Thr-66 resides in the core of the protein, located on the central β -sheet F (as labeled for human PEBP; Banfield et al., 1998). The Thr side chain forms a hydrogen

Mutant Line	Position	Nucleotide Substitution	Major Effect
det-1	+202 (junction exon 1/intron 1)	TG G T → TG A T	Intron 1 is not spliced
det-2	+12 (exon 1)	$ATG \to ATC$	$Met-4 \rightarrow IIe$
	+139 (exon 1)	$ACC \rightarrow CCC$	Thr-47 \rightarrow Pro
	+401 (intron 2)	$AACCA \rightarrow AATCA$	Silent
	+655 (exon 4)	$CAA \rightarrow CGA$	GIn-127 $ ightarrow$ Arg
	+749 (exon 4)	$GTT \rightarrow GTG$	Silent
det-3	+12 (exon 1)	$ATG \to ATC$	$Met-4 \rightarrow IIe$
	+197 (exon 1)	$ACA \rightarrow ATA$	Thr-66 $ ightarrow$ lle
	+401 (intron 2)	$AACCA \rightarrow AATCA$	Silent
	+584 (exon 4)	$\mathbf{G} A G ightarrow \mathbf{A} A G$	$Glu-104 \rightarrow Lys$
	+749 (exon 4)	$GTT \rightarrow GTG$	Silent

The positions of the substitutions are labeled from the +1 of translation. For *det-1*, the sequence is compared with the progenitor line, Paloma, whereas for *det-2* and *det-3*, the sequence is compared with the wild-type line Térèse. "Silent" indicates that the substitution has no effect at the amino acid sequence of the protein. Mutations in boldface are proposed to be responsible for the *det* phenotype (see details in the text).

bond to the side chain of Gln-127. Therefore, these two residues (Thr-66 and Gln-127) map to the same region of the structure. Interestingly, two of the *det* mutants, *det-2* and *det-3*, represent mutations in these residues. These mutations (Thr-66 to lle in *det-3* and Gln-127 to Arg in *det-2*) would disrupt the hydrogen bonding interaction between the two residues and likely would affect the structural integrity of this region. Because mutations that affect protein function have been mapped to this region in both Arabidopsis TFL1 and PsTFL1a, it follows that this must be an important part of the structure. However, this importance is likely to be structural (maintenance of protein fold) rather than directly functional (e.g., interaction with other proteins/small molecules).

Because three different *det* mutant alleles carry a substitution in the predicted amino acid sequence of *PsTFL1a* that is likely to have a significant effect on PsTFL1a function, we conclude that *PsTFL1a* corresponds to *DET*.

PsTFL1c Is Another Homolog of TFL1 and Corresponds to LF

We also examined the relationship between *PsTFL1c* and *LF* by sequencing *PsTFL1c* from a range of natural variant alleles and induced mutants at the *LF* locus (Table 2). A relatively large number of *LF* alleles are known and have been grouped into four phenotypic classes (Taylor and Murfet, 1993; Weller et al., 1997). Mutants in the *If-a* class present the strongest phenotype and can flower as early as node 5. In four of six independent *If-a* lines—HL7, XVIII/17, Wt11796, and K2—*PsTFL1c* could not be amplified from genomic DNA (data not shown), and a band corresponding to *PsTFL1c* was absent from these lines in DNA gel blot analyses (Figure 5). These lines were obtained from fast-neutron or γ -ray mutagenesis (Taylor and Murfet, 1993) and therefore likely carry large deletions in the region of *PsTFL1c*.

In the remaining two *lf-a* mutants (HL76 and Wt11795), *PsTFL1c* was amplified and sequenced (Table 2, Figure 6). Compared with its isogenic *Lf* progenitor HL75, the *lf-a* line HL76 was found to carry a 6-bp deletion in the *PsTFL1c* coding region that is predicted to direct the replacement of three

amino acids (Phe-Ala-Asp) at positions 147 to 149 with a single Tyr (haplotype B in Figure 6). This deletion maps to the α -C helix (as labeled for the human PEBP [Banfield et al., 1998]), and in the mutated form, it is highly unlikely that this helix would be able to form. Loss of this helix would have a seriously destabilizing effect on the protein's structure. Interestingly, this helix maps to the same region of the structure as the Thr-66/Gln-127 pair, again suggesting the importance of this area in protein stability and/or function. In the *If-a* line Wt11795, a C-to-T substitution at position 1625 is responsible for the replacement of Pro-109 by Ser (haplotype E in Figure 6). This Pro is conserved in all TFL1-related plant sequences (Figure 2A) and forms an integral part of the proposed "putative ligand binding site" for these proteins (Banfield et al., 1998). Consequently, this mutation could affect protein function severely.

Our analysis of six independent *lf-a* mutants has shown in each case an important modification in the *PsTFL1c* sequence, either a large deletion or a functionally significant substitution mutation. Therefore, we conclude that *PsTFL1c* corresponds to *LF*.



Figure 4. Analysis of the Nonsplicing of the First Intron in the *det-1* Line.

PCR was performed on root cDNA from Paloma (Pa) and *det-1* (JI 2121) with water (H₂O) and genomic DNA (gDNA) as controls. The primer pairs 5R*1R5 and 5R*5R1 surrounded intron 1 and introns 1 and 2, respectively. Arrowheads indicate the size expected for the cDNA if the first intron is spliced (cDNA) or not spliced (cDNA+intron 1). A 100-bp size marker lane is shown in the center of the gel.

Line	LF Class	Haplotype	Derived Mutant Line	LF Class	Haplotype
Vesna	Lf-d	А	XVIII/17	lf-a	No
Torsdag	Lf	А	K319	lf	С
			K2	lf-a	No
Wt4042	Lf	А	Wt11796	lf-a	No
HL75	Lf	А	HL76	lf-a	В
Porta	Lf	А	Wt11790	lf	С
			Wt11791	lf	С
Paloma	Lf	А	Wt11795	lf-a	E
WL1771	Lf-d	D	WL1770	Lf	D
			WL1769	lf	D
Champagne	Lf-d	А			
Térèse	Lf	А			
			HL7	lf-a	No

Mutants and their progenitor lines are described by Taylor and Murfet (1993). The *PsTFL1c* sequences were grouped in five haplotypes (A to E) as described in Figure 6. "No" indicates that *PsTFL1c* could not be amplified by PCR and was not detected on a DNA gel blot (Figure 5). HL7 is a natural mutant; therefore, no progenitor line is available.

The Three *PsTFL1* Genes Present Different Patterns of Expression

We next examined the presence/absence of transcript for each of the *PsTFL1* genes by RT-PCR in different tissues at different stages. Specific intron-spanning primers were designed for each gene to control for contaminating genomic DNA. We analyzed a number of different tissues during the vegetative and reproductive phases in plants grown under either short-day or long-day conditions (Figure 7). The three genes showed distinct patterns of expression. *PsTFL1a* was expressed mainly in roots, in the apex after the floral transition, and in flower buds and flowers. Expression in roots is common for flowering genes such as *LpTFL1* (Jensen et al., 2001), *LUMINIDEPENDENS* (Aukerman and Amasino, 1996), and *GIGANTEA* (Fowler et al., 1999). No function was proposed for the expression of these

Figure 5. DNA Gel Blot of Different Wild-Type and Corresponding Mutant Lines for *LF*.

Mutant lines are described in Table 2. The genomic DNA was digested with EcoRI, and the blot was hybridized with a *PsTFL1c* probe under high stringency.

genes in roots. No expression of *PsTFL1a* was detected in the shoot apex before the floral transition. In both short-day and long-day conditions, a signal was detected in the apex after the floral transition (stage A2) and remained during the reproductive phase (stage A3). For *PsTFL1b*, expression was found in the apex during the vegetative and reproductive phases (Figure 7). Expression was found in roots and dormant nodes. No expression was detectable in flowers. As with *PsTFL1a*, no difference was observed between short-day and long-day conditions. *PsTFL1c* transcripts were present in all tissues studied (Figure 7), and no change in the expression pattern was detected during the flowering process.

Intermediate Mutant Lines for *LF* Present Variations of the *PsTFL1c* Transcript Level

As shown previously, the stronger *LF* alleles (*If-a*) carry deletions or substitutions of the *PsTFL1c* gene. Other alleles (*If, Lf,* and *Lf-d*) were studied (Table 2). DNA gel blot analysis revealed no detectable structural change for some of these lines (K319 and WL1769; Figure 5). Sequence analysis showed no differences in the coding region. Only two silent substitutions in the introns were detected: one between *If* mutants K319, Wt11790, and Wt11791 (haplotype C) and their wild-type progenitors (haplotype A) and another between the wild-type line WL1771, its derived mutant lines (haplotype D), and the other wild-type lines (haplotype A) (Table 2, Figure 6). Therefore, plants containing the *Lf-d*, *Lf*, or *If* allele produce the same PsTFL1c protein.

To determine whether differences between LF alleles occurred at the transcriptional level, PsTFL1c transcripts were analyzed by real-time PCR in the apices of plants with five expanded leaves (Figure 8). At this stage, plants have not initiated flowering, except those bearing the strong *lf-a* allele. In the mutant series WL1770 (*Lf*) and WL1769 (*lf*), derived from the WL1771 (*Lf-d*) progenitor line (Table 2), *PsTFL1c* was expressed sequentially higher. Between *Lf-d* and *lf*, there was a





Figure 6. Description of the Different Haplotypes Identified from the Sequence Analysis of *PsTFL1c* in Several Pea Lines and Their Corresponding Mutants for *LF*.

Mutant lines are described in Table 2. Scheme of the *PsTFL1c* genomic sequence. Boxes represent the coding sequence. Arrows indicate the positions of the substitutions relative to haplotype A. For each haplotype, the position and the nature of each substitution are described and the predicted effect on the amino acid sequence is given.

10-fold difference (Figure 8). Therefore, for a given genetic background, there was a correlation between the expression of *PsTFL1c* and the node of flowering. A similar correlation was found in mutants K319 (*If*) and K2 (*If-a*) in the Torsdag (*Lf*) background. *PsTFL1c* transcript levels were threefold higher in Torsdag than in K319 (*If*), whereas no significant expression was detected in K2, which carries a large deletion spanning the *TFL1* gene (Figure 6).

These results suggested that the low level of *PsTFL1c* transcription was associated with early flowering. This correlation seemed to be contradicted by the analysis of HL76 (*If-a*), in which a relatively high level of *PsTFL1c* transcript was associated with early flowering. The *PsTFL1c* transcript level was not significantly different between HL75 (*Lf*) and HL76 (*If-a*) (Figure 8). This contradiction can be explained by the presence of a mutation in the predicted PsTFL1c protein of HL76 (Figure 6). For this line, protein sequence, not mRNA level, was the cause of the early-flowering phenotype. Because no change was detected in *PsTFL1c* transcript level between HL75 and HL76, this finding suggests that there is no feedback regulation for *LF*.

We noticed that within the same class of *LF* allele, there was variation in the level of *PsTFL1c* transcript. For example, for the *Lf* allele, the relative expression level varied from 67 (Térèse line) to 151 (Torsdag line). This variation could be a consequence of modulation resulting from different genetic backgrounds. To test this hypothesis, we introduced *LF* alleles in new genetic backgrounds by successive backcrosses. The *Lf-d* allele, carried by the HL66 line, was introduced into the Térèse background by seven successive backcrosses. In this new genetic background, the level of *PsTFL1c* transcript in *Lf-d* plants was 40% lower than that in HL66 but still significantly higher (threefold) than that in the nearly isogenic *Lf* line Térèse (Figure 8). From these results, we conclude that *PsTFL1c* transcription is determined by the *LF* allele but also by different genetic backgrounds.

DISCUSSION

The genetics and physiology of flowering in pea have been studied in detail (Reid et al., 1996; Weller et al., 1997). We used a candidate-gene approach to study the role of *TFL1* homologs in the flowering process. Two of these homologs, *PsTFL1a* and *PsTFL1c*, were found to correspond to the pea genes *DET* and *LF*, respectively. Evidence for these correspondences and the conservation of *TFL1* homolog gene function between Arabidopsis and pea are discussed below.

DET, an Arabidopsis *TFL1* Homolog, Acts to Maintain the Fate of the Inflorescence Meristem in Pea

PsTFL1a mapped in the vicinity of *DET*. Sequencing of *PsTFL1a* in three independent *det* lines revealed mutations that would significantly modify PsTFL1a structure, leading to a non-functional protein. These results strongly indicate that *PsTFL1a* corresponds to *DET*. Further evidence that *PsTFL1a* corresponds to *DET* comes from mutant phenotype comparisons and gene interactions.

The phenotype of *det* mutants in pea is similar to those of *tfl1* and *cen*. In all three species, indeterminate growth is changed to determinate growth during the reproductive phase (Singer et al., 1990; Bradley et al., 1996, 1997). This conversion was proposed to result from the acceleration of the reproductive phase and the conversion of the inflorescence to a floral meristem in Arabidopsis (Ratcliffe et al., 1998). In *det* mutants, the inflorescence meristem is converted to a stub (Singer et al., 1999). As with *cen* mutants in snapdragon, *det* mutants present no phenotype during the vegetative phase, whereas *tfl1* mutants flower earlier in Arabidopsis. The absence of a vegetative phe-



Figure 7. Expression Analysis of Pea TFL1 Homologs by RT-PCR.

Specific primers for *PsTFL1a*, *PsTFL1b*, and *PsTFL1c* were used to amplify the cDNA (see Methods). As controls, PCR was performed on genomic DNA (gDNA) and water (0). PCR was performed on cDNA obtained from different tissues: root (R), dormant node 4 (N), internode (IN), leaf (L), vegetative apex (A), flower bud (FB), flower (F), and shoot apex during the floral transition under short-day (SD) or long-day (LD) conditions at three different stages (A1, before the floral transition; A2, after the floral transition but before flowering; and A3, after flowering). These three stages are described in detail in Methods.



Figure 8. Analysis of the PsTFL1c Transcript Level in Different LF Lines by Real-Time PCR.

RNA was extracted from the apices of plants at the five-node stage. To compare the results, transcript levels also were evaluated for the elongation factor, $E1F\alpha$, which is supposed to be constant in our conditions. Numbers represent the differences in expression between *PsTFL1c* and *EF1\alpha*. The real-time PCR experiment was repeated three times. For each *LF* line, the allele at the *LF* locus and the node of flowering (average of four different plants grown in short-day conditions) are given. V indicates that the plants did not flower after 40 nodes and remained vegetative.

notype may be explained by the fact that *DET* and *CEN* (Bradley et al., 1996) are expressed only after the floral transition, whereas *TFL1* also is expressed during the vegetative phase (Bradley et al., 1997).

Interactions between *TFL1* and other important regulatory genes are conserved between some species. In Arabidopsis, *TFL1* antagonizes *LFY*, as does *CEN* with *FLORICAULA* in snapdragon. Double mutants (*tf11 lfy* or *cen flo*) have a *lfy* or *flo* phenotype, respectively (Bradley et al., 1996, 1997). The same epistatic interaction is found in pea between *DET* and *UNIFOLI-ATA*, the *LFY* ortholog. The double mutant *det uni* has a *uni* phenotype (Singer et al., 1999).

LF, Another Arabidopsis *TFL1* Homolog, Is a Repressor of Flowering

The results obtained demonstrate that *PsTFL1c* corresponds to *LF*. *PsTFL1c* was mapped to linkage group II in the vicinity of *LF*. Analysis of *PsTFL1c* genomic DNA sequence in the strongest *If-a* mutants (plants presenting the earliest flowering phenotype) showed significant modifications: four *If-a* mutants are complete deletion mutants lacking *PsTFL1c* and can be considered null alleles; two other *If-a* mutants contained nonsilent changes that could modify the structure or function of PsTFL1c.

Comparison of the *lf-a* and *tfl1* mutants reveals a similar early-flowering phenotype. In Arabidopsis, *TFL1* acts to maintain the apical meristem during the vegetative stage and thereby control the length of the vegetative phase (Bradley et

al., 1997; Ratcliffe et al., 1998). In pea, *LF* also can be considered a regulator of the length of the vegetative phase. Mutant lines for this gene have a lower flowering node and early flowering time (Taylor and Murfet, 1993). *LF*, like *TFL1*, acts as a floral repressor by lengthening the vegetative phase. In contrast to tf/1, no major floral phenotype was detected for mutants at the *LF* locus.

Natural and induced mutant alleles for LF have been grouped into four classes: Lf-d, Lf, If, and If-a (Taylor and Murfet, 1993). The strongest *lf-a* phenotype is the result of mutation or deletion of PsTFL1c. This loss of function is consistent with If-a being a recessive mutation. Sequencing of the Lf-d, Lf, and If alleles revealed no change in the predicted amino acid sequence of PsTFL1c (Table 2, Figure 6). We demonstrated that the flowering phenotype in these alleles could be explained by differences in PsTFL1c transcript levels during the vegetative phase in the shoot apex. When comparing series of alleles in the same genetic background, LF transcript levels were correlated with the flowering node in pea. Plants having the late-flowering Lf-d allele had a high level of LF transcript. The Lf-d plants behaved like 35S-TFL1 Arabidopsis plants, in which the upregulation of TFL1 is responsible for a delay in flowering and a highly branched architecture (Ratcliffe et al., 1998). In pea, the influence of flowering genes on branching is well known (Floyd and Murfet, 1986; Beveridge et al., 2003). In particular, the Lf-d allele, by delaying flower initiation, results in plants with a high number of aerial lateral branches. The high transcript levels in Lf-d plants are consistent with the dominance of this allele. Furthermore, a transcript dose effect also would explain the intermediate flowering node seen in heterozygous individuals (Murfet, 1975).

One hypothesis to explain the differences in transcript levels between the LF alleles is that important cis-regulatory elements are mutated. We have sequenced the untranslated region (200 bp 5' and 260 bp 3') and the introns in a series of different LF alleles (Table 2), and only two changes were detected. One substitution (haplotype D; Figure 6) seems to result from natural variation with no effect on flowering, because it is found in WL1771 (Lf-d), WL1770 (Lf), and WL1769 (If) (Table 2). More interesting is the substitution in intron 1 (haplotype C; Figure 6), which is found in independent *If* plants derived from different wild-type lines. If lines Wt11790 and Wt11791 were obtained from the Lf line Porta (Murfet, 1991), whereas If line K319 was obtained from the Lf line Torsdag (Uzhintseva and Sidorova, 1988). It is intriguing that identical alleles appeared independently in different mutagenesis programs. Important cis elements can be present in the introns, as was shown in the second intron of AGAMOUS (Lohmann et al., 2001). Modification of the expression of genes between different alleles has been demonstrated for important traits. In tomato, the fw2.2 alleles regulate fruit size through changes in transcript regulation rather than in the FW2.2 protein itself (Cong et al., 2002). In rice, Hd3, a FT homolog, shows different levels and timing of gene expression between different alleles (Kojima et al., 2002). In both cases, SNP between the alleles have been detected in putative regulatory regions. Further analysis will be required to prove the association between SNP and expression changes in the If alleles.

Different Regulation between *TFL1* Homologs in Pea and Arabidopsis

In pea, two TFL1 homologs have two distinct functions: LF is involved in the control of the vegetative phase by delaying floral initiation, the transition from the vegetative to the I1 inflorescence meristem, and *DET* is involved in the control of the floral phase by preventing the transition from the I1 inflorescence meristem to the flower (Figure 9). This regulation is different from that in Arabidopsis, in which only one TFL1 gene controls the length of both the vegetative and floral phases (Figure 9). To obtain the tfl1 phenotype in pea (early flowering and determinate growth), a det If double mutant is necessary (Murfet, 1989). LF and DET are homologs and may derive from a common ancestor by duplication. In pea, the two genes may have evolved separately and become specialized for two distinct functions. The det If-a double mutant has only additive effects and no extra phenotype (Murfet, 1989), which suggests nonredundant functions for DET and LF. It has been argued that subfunctionalization of duplicated genes is a mechanism whereby degenerative mutations can lead to the preservation of duplicated genes (Force et al., 1999; Lynch and Force, 2000). DET and LF may provide an example in which a gene that was expressed originally during both the vegetative and reproductive phases (such as TFL1 in Arabidopsis) diverged into two copies with a partitioning of gene expression patterns as predicted by the duplication/degeneration/complementation model. Like DET, CEN has only one function during the vegetative phase in



Figure 9. Model for the Control of Floral Initiation and Development by *TFL1* Genes in Arabidopsis and Pea.

F, flower; I1, inflorescence meristem; V, vegetative phase.

snapdragon (Bradley et al., 1996), so it will be interesting to determine whether another *TFL1* homolog exists in snapdragon that functions during the vegetative phase in a manner similar to *LF*.

In this study, we have demonstrated the important role played by a TFL1 homolog, LF, in the control of flowering time in pea. Detailed genetic analyses have enabled the identification of a few genes involved in the natural variation in flowering time among pea cultivars under controlled environmental conditions (Murfet, 1971b; Weller et al., 1997). LF was identified as a major contributor to natural variability, but other genes involved in the photoperiodic pathways also were shown to influence flowering time in pea, such as STERILE NODE and HIGH RESPONSE. These genes are implicated in the synthesis of a graft-transmissible floral inhibitor, which is perceived in the apex by LF (Murfet, 1971a). As a long-day flowering plant, pea has developed a strategy to control flowering in response to photoperiod and vernalization, with a central role for LF. Vernalization acts quantitatively by reducing the flowering node in two ways: by decreasing inhibitor production and by rendering the apex more sensitive to the flowering signal (Murfet and Reid, 1974; Reid and Murfet, 1975).

The strategy developed by Arabidopsis seems to be different, because TFL1 plays a minor role in the control of flowering time between ecotypes. In Arabidopsis, FLOWERING LOCUS C (FLC) and FRIGIDA (FRI) are central elements and are key components of the response to vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). For instance, in natural Japanese Arabidopsis populations, the digenic interaction between FRI and FLC was the major genetic system controlling flowering time (Shibaike et al., 1999). FLC is a floral repressor, which must be repressed by vernalization for floral initiation to occur. FRI acts synergistically by maintaining high levels of FLC. Early-flowering ecotypes of Arabidopsis carry nonfunctional FRI and/or FLC alleles (Johanson et al., 2000) and consequently have low levels of FLC transcript, whereas late-flowering ecotypes have high levels of FLC expression (Rouse et al., 2002). This important role for FLC also is found in other Brassicaceae species (Tadege et al., 2001; Schranz et al., 2002). Thus, pea and Arabidopsis have developed different strategies to control flowering time, which may represent different strategies to respond to environmental conditions. In natural Arabidopsis populations, flowering time variation is explained mainly by response to vernalization, whereas in pea it seems to be explained mostly by response to photoperiod. Interestingly, in both cases, the transcript level of the repressor, FLC or LF, determines flowering time. Different alleles exist at the *LF* locus and are responsible for different *LF* transcript levels. The same results were found in Arabidopsis at the *FLC* locus, where different alleles confer different *FLC* transcript levels (Schlappi, 2001).

In conclusion, we have shown that *TFL1* homologs in pea play an important role in floral initiation and development. LF is a major gene that controls flowering time by integrating different environmental and endogenous signals. Understanding the regulation of LF by genes involved in photoperiod response is an important next step for dissecting the molecular basis of flowering control in pea.

METHODS

Plant Material and Growth Conditions

det lines of pea (*Pisum sativum*) were obtained from the John Innes Pisum Germplasm collection. *det-1* corresponds to JI 2121 and was obtained by mutation of cv Paloma (Swiecicki, 1987). *det-2* (JI 1358) is a spontaneous mutant, and *det-3* (JI 3100) was obtained by mutation of line SG (Berdnikov et al., 1999). Other pea cultivars were Térèse and Torsdag. The F2 and RI lines used for genetic mapping are described below. *LF* lines were provided by Ian Murfet (University of Tasmania, Hobart, Australia). Relevant information concerning the mutants and their progenitors is described by Taylor and Murfet (1993).

For the floral initiation experiments, the plants were grown in cabinets at 20°C during the day and 15°C during the night with illumination by mercury vapor lamps (135 mE·m⁻²·s⁻¹) in a 1:1 mixture of sphagnum: clay under short-day (12 h of light) or long-day (18 h of light) conditions. For the long-day conditions, plants received 12 h of light from the mercury vapor lamps extended by 6 h of light from a series of 40-W incandescent/fluorescent bulbs. The apices were harvested at three different stages. The A1 stage was collected at least 10 nodes before plants flower. At this stage, the apices are still vegetative (Isabelle Lejeune, personal communication). The A2 stage was harvested just three nodes before flowering. At the A3 stage corresponds to the apices just after flowers open.

Cloning and Isolation of Genes

The *TFL1* homologs were isolated in cv Térèse using degenerate primers. The PCR conditions were 35 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 2 min. The primer combination TFL1-1 (5'-ATGGGG-AGAGTGATA/T/CGGG/AGAA/TG-3') and TFL1-2 (5'-TCACTAGGA/ GCCA/TGGAACATCA/TGG-3') gives a 450-bp band corresponding to *PsTFL1c*. Using the primer combination TFL1-3 (5'-GATGTTCCA/ TGGA/TCCTAGTGAC/TCC-3') and TFL1-5 (5'-CTTGCAGCA/GGTC/TC/ TTCC/TCTC/TTG-3'), two bands of 400 and 800 bp were obtained corresponding to *PsTFL1a* and *PsTFL1b*, respectively.

The sequences of the genes were extended by 3' rapid amplification of cDNA ends (RACE) PCR using the kit and the recommendations of the supplier (Life Technologies, Rockville, MD) on cDNA from seedlings and flowers. The following primers were used as gene-specific primers and nested gene-specific primers, respectively, for *PsTFL1a* (TFL1R1 [5'-TCAAACAAAGAGCGAGAGATTCAG-3'] and TFL1R2 [5'-AGACCA-TTTCAACACTCGTAG-3']), for *PsTFL1b* (Tb31 [5'-GACAGATATACC-AGGCACAAC-3'] and Tb32 [5'-GAAATATGAAATGCCACGTCC-3']), and for *PsTFL1c* (TFL1-1 and TFL5R3 [5'-AGCCAAGGATTCAGA-TTCAAGG-3']). The 5' part of *PsTFL1a* was obtained by 5' RACE PCR using the kit and the recommendations of the supplier (Life Technologies) on cDNA from flowers. The gene-specific primers used were TFLR3 (5'-CTACTTTGATACACACACGACAC-3') and TFL5R1 (3'-GAATAGAAC-AAACACAAACCT-3'). The 5' part of *PsTFL1c* was recovered by PCR walking on genomic DNA (Devic et al., 1997). Pea genomic DNA was digested by Dral, and adaptors were ligated to the digested DNA as described by Devic et al. (1997). The following specific primers were used to amplify the gene: TFL1cR8 (5'-TAACTGTAGAAGGAAAGGGTA-3') and TFL1cR9 (5'-ATGCTTGCGGTAAAATAATCA-3').

Mapping and Marker Development

Mapping was performed on two different mapping populations (recombinant inbred lines) obtained from the crosses Térèse × K586 (Laucou et al., 1998) and JI 281 × JI 399 (Ellis et al., 1992). Polymorphisms were sought between the parents in the mapping population to develop PCR markers such as cleaved amplified polymorphism sequence (CAPS) markers. For *PsTFL1a*, a single nucleotide polymorphism was detected between Térèse and K586. A derived CAPS (dCAPS) marker (Neff et al., 1998) was developed using the following primers (TFL2, 5'-GAACACTTG-CACTGGTAAATATAATAGA-3'; TFLR, 5'-TGTAGCATCTGTTGTTCC-TGG-3') and the Hinfl enzyme. For *PsTFL1b* and *PsTFL1c*, polymorphisms were found between JI 281 and JI 399 and CAPS markers were developed for *PsTFL1b* (primer pairs TFLb32 [5'-GAAATATGAAATGCC-ACGTCC-3'] and TFL1bR1 [5'-ACAAACTAGAACAACAACAACCACCC-3'] and restriction enzyme Hhal) and for *PsTFL1c* (primer pairs TFL1-1 and TFL1-2 and restriction enzyme HpyF44III).

DNA Extraction and Gel Blots

DNA was extracted from leaves according to the protocol described by Laucou et al. (1998). Ten micrograms of genomic DNA was digested with restriction enzymes and loaded on a 0.7% agarose gel. Blotting was performed according to the recommendation of the membrane supplier (Biotrans Nylon Membrane; ICN, Costa Mesa, CA). Hybridization was performed in Church and Gilbert (1984) buffer at 65°C overnight. Washing was done at 65°C according to Sambrook et al. (1989) (2× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS for 30 min, 1× SSC and 0.1% SDS for 15 min, and 0.2× SSC and 0.1% SDS for 15 min).

RNA Extraction and Expression Analysis

RNA was extracted using the RNeasy Plant Mini Kit with a DNase treatment on an RNeasy column (Qiagen, Valencia, CA). Five micrograms of total RNA was used for cDNA synthesis. Before reverse transcription, total RNA was treated with amplification-grade DNase I (Life Technologies) according to the manufacturer's protocol. Reverse transcription was performed using 200 units of Superscript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA) in the presence of 40 units of recombinant ribonuclease inhibitor (Life Technologies) with the AP primer. For PCR, cDNAs were resuspended in 100 μ L of water, and 1 μ L was used per reaction. Reverse transcriptase-mediated PCR was performed using specific primers for PsTFL1a (TFL5RACE [5'-TGAGTTGTACTCTTA-AGTTCTTC-3'] and TFLaR5 [5'-AGGGCCAGGAACATCAGGGTC-3']), PsTFL1b (TFL1bR1 and TFL1b31 [5'-GACAGATATACCAGGCACAAC-AG-3']), and PsTFL1c (TFLcR2 [5'-AAATAAGCAGCAGCAACAGGG-3'] and TFLcR3 [5'-CAGACATTCCAGGGACAACAG-3']) and the following program (40 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s). The PCR product was loaded on a 2% (w/v) agarose gel.

Real-time PCR was performed on a Roche Lightcycler using the Fast-Start DNA Master SYBR Green I kit (Mannheim, Germany) according to the manufacturer's protocol. Specific primers suitable for quantitative reverse transcriptase-mediated PCR were designed using LC Probe Design Report software (Roche) for *EF1* α (E1FF1 [5'-GATGCACCT-GGACATCGTGACT-3'] and E1FR1 [5'-CTTAGGGGTGGTAGCATCCAT-CT-3']) and *PsTFL1c* (cF3 [5'-CCACATTTGGAAAAGAGTTGACAA-GC-3'] and cR3 [5'-GCGTCTTCTAGGAGCCGTTGC-3']). The PCR program consisted of a first step of denaturation and Taq activation (95°C for 8 min) followed by 45 cycles of denaturation (94°C for 10 s), annealing (58°C for 7 s), and extension (72°C for 10 s). At the end, amplified products were denatured (95°C), renatured (65°C), and progressively denatured (step from 65 to 95°C over 30 min or 0.1°C/s for the fusion curve analysis). Both primers combinations (cR3/cF3 and E1FR1/E1FE1) were tested (fusion curve, linearity, and efficiency of the primers). The primer combinations for *E1F* and *PsTFL1c* have a PCR efficiency of 86 and 80%, respectively.

For PCR, cDNA was diluted 50 times and 5 μ L was used as a template in a 20- μ L reaction mix. *PsTFL1c* transcript level was estimated based on the level of the constitutive *EF1* α gene (Nesi et al., 2000). For each condition, the number of cycles necessary to reach a certain level (exit position) of fluorescence was evaluated for *EF1* α (n_{EF1 α}) and *PsTFL1c* (n_{PsTFL1c}). The difference between the exit points of the two genes was calculated (d1 = n_{PsTFL1c} – n_{EF1 α}). The value 2^{d1} represents the difference of copy number between *PsTFL1c* and the constitutively expressed gene, *EF1* α . Because *EF1* α was expressed at a higher level than *PsTFL1c*, the final ratio was calculated as follows: PsTFL1c level = (1/2^d) × 100,000% EF1 α .

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Catherine Rameau, rameau@versaille.inra.fr.

Accession Numbers

The sequence data described herein have been submitted to GenBank with accession numbers AY340579 for *PsTFL1a*, AY340580 for *PsTFL1b*, and AY343326 for *PsTFL1c*. Accession numbers for the sequences shown in Figure 2 are as follows: AB027506 (TSF), AB016880 (BFT), AF147721 (MFT), and BAA33415 (*BnTFL1-1* from *Brassica napus*).

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