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Linalool and linalool nerolidol synthases in roses, several genes for little scent.

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Abstract

Roses are widely appreciated for the appearance of their flowers and for their fragrance. This latter character results from the combination of different odorant molecules among which monoterpenes are often prevalent constituents. In this study, we report the cloning and characterization of three rose monoterpene synthases. In vitro functional characterization of these enzymes showed that one is a (-)-(*3R*)-linalool synthase whereas the others have a dual (+)-(*3S*)-linalool nerolidol synthase activity. However, given that the characterized rose cultivars were only able to produce the (-)-(*3R*)-linalool stereoisomer, the linalool nerolidol synthases are probably not active in planta. Furthermore, these three enzymes were also characterized by a weak expression level as assessed by RT-qPCR and by the low abundance of the corresponding sequences in an EST library. This characteristic is likely to explain why linalool is generally a minor constituent in rose flowers' scents. On this basis, we propose that in roses the monoterpene biosynthesis effort is focused on the production of acyclic monoterpenes derived from geraniol through the recently characterized Nudix biosynthesis pathway, at the expense of conventional monoterpene biosynthesis via terpene synthases such as linalool or linalool nerolidol synthases.

Keywords:
Rosa chinensis; Flower fragrance; Monoterpene biosynthesis pathways; Terpene synthases; (-)-(*3R*)-linalool; (+)-(*3S*)-linalool; nerolidol

Abbreviations:
DMAPP: dimethylallyl diphosphate, EST: expressed sequence tag, FPP: farnesyl pyrophosphate, GFP: green fluorescent protein, GP: geranyl monophosphate, GPP: geranyl pyrophosphate, IPP: isopentenyl diphosphate, RPKM: reads per kilobase per million, TPS: terpene synthase

1. Introduction

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A number of different monoterpenes participate in the typical fragrance of roses. In most plants, monoterpenes are synthesized in the plastidial compartment and derive from production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through the methyl erythritol phosphate pathway. IPP and DMAPP are subsequently condensed into geranyl diphosphate (GPP) by a geranyl diphosphate synthase (Chen et al., 2011). The GPP is further transformed into diverse monoterpenes through the activity of different plastidial monoterpene synthases. Catalysis by terpene synthases (TPS) starts by the removal of the pyrophosphate group of GPP leading to a carbocation, which is subsequently rearranged, prior to the termination of the reaction upon neutralization of this carbocation. Depending on whether monoterpene synthases promote the cyclization of the substrate or not, cyclic (e.g. limonene) or acyclic (e.g. linalool) monoterpenes will be produced (Degenhardt et al., 2009). A parallel pathway for sesquiterpene synthesis takes place inside the cytosolic compartment: two IPP and one DMAPP molecules produced through the mevalonate pathway are condensed by a farnesyl pyrophosphate synthase into farnesyl pyrophosphate (FPP). FPP is further transformed into sesquiterpenes following the activity of cytosolic sesquiterpene synthases (Chen et al., 2011). In roses however, the synthesis of acyclic monoterpenes has recently been shown to proceed through a specific pathway (Magnard et al., 2015). This pathway involves RhNUDX1, an enzyme of the Nudix hydrolase family. Nudix hydrolases generally cleave nucleoside diphosphate linked to other moieties (Bessman et al., 1996). However, some of them, such as RhNUDX1 would accept non-nucleoside substrates. Indeed, RhNUDX1 is able to dephosphorylate GPP into geranyl monophosphate (GP). The resulting GP is probably further dephosphorylated into geraniol by a yet unknown phosphatase. Requirement in a cytosolic pool of GPP for monoterpene production is another important difference between roses and other plants since RhNUDX1 has a cytosolic location. GPP synthesis through the cytosolic mevalonate pathway has been suggested (Dunphy, 2006), although transport from the plastid can also be hypothesized (Gutensohn et al., 2013). The main difference between the synthesis of monoterpenes through the conventional (involving terpene synthases) or Nudix pathways lies in the ability of TPS to generate a reactive carbocation that can be modeled in the enzyme's active site in order to generate different terpenic skeletons, including a cyclic one (Degenhardt et al., 2009). Due to the number of existing monoterpene synthases and the ability of many of them to generate several products, an extensive range of different monoterpenes can be obtained. On the contrary, the RhNUDX1 hydrolase acts via a dephosphorylation mechanism, which excludes reorganization of the substrate, especially its cyclization. Consequently, only geraniol whose carbohydrate skeleton is similar to that of GPP is produced. However, enzymes able to transform geraniol into other acyclic monoterpenes have been characterized in some plants. In basil, ginger, perilla and orchid, dehydrogenases transform geraniol into geranial, and possibly neral into nerol (neral being probably obtained by spontaneous isomerization of geranial) (Iijima et al., 2006; Sato-Masumoto and Ito, 2014; Xu et al., 2017). In orchid a geranial reductase produces citronellal, which can be further isomerized into citronellol by the same dehydrogenase as that involved in geraniol for geranial conversion (Xu et al., 2017). Similar enzymes could possibly be active in rose petals. However, despite further isomerization of geraniol, the number of resulting products is reduced compared to the conventional pathway involving terpene synthases.

In roses tested so far, extensive amounts of geraniol and derivatives are produced through the Nudix pathway (Magnard et al., 2015). One reason is certainly the high affinity of RhNUDX1 for GPP, its Km for this compound being 140 nM. In comparison, the Km of the conventional geraniol synthase from basil is 21 µM (Iijima et al., 2004). Consequently, the question arises whether the GPP stock present in roses' petal cells is fully used by RhNUDX1 or whether some monoterpenes are still produced via conventional TPS. Indeed, compared to

geraniol and derivatives, other acyclic (linalool, myrcene, ocimene) or cyclic (limonene, pinene) monoterpenes are rarely found in roses or only found in low quantities. Furthermore, to our knowledge no enzyme with a proven monoterpene synthase activity has been characterized in roses so far.

To gain some insight into the question of the relationship between each monoterpene biosynthesis pathways, we tried to characterize conventional terpene synthases expressed in rose flowers. Towards this aim, we took advantage of an existing expressed sequence tag (EST) database produced from a wide range of organs and developmental stages of the *Rosa chinensis* cultivar 'Old Blush' (Dubois et al., 2012). This database was searched for sequences presenting homologies to monoterpene synthases isolated in other species. Only four sequences were recovered from the whole database, including one which proved to be a pseudogene lacking a significant part of the 5' extremity. In vitro enzymatic activity indicated that the other genes corresponded to linalool or linalool nerolidol synthases clustering in the TPS-b and TPS-g subfamilies of terpene synthases. In order to establish a correlation between these enzymes and scent production in roses, linalool accumulation in flower tissues of different cultivars at different developmental stages was measured. In parallel, the expression of the cloned enzymes was quantified in flower tissues by quantitative RT-PCR. Together with the characterization of the type of linalool stereoisomers they produce, this allowed to assess the respective implications of these enzymes in fragrance production in rose flowers.

2. Material and Methods

2.1. Rose cultivars

Rose plants were grown in field at Saint-Etienne University (*Rosa chinensis* cultivar 'Old Blush' and *Rosa* x *hybrida* cultivar 'The McCartney rose') and in Lyon at Ecole Normale Supérieure (*Rosa* x *hybrida* cultivar 'Lady Hillingdon'). Flower developmental stages are presented on Figure 5A.

2.2. Cloning of *Rosa chinensis* 'Old blush' linalool and linalool/nerolidol synthases

'Old Blush' genomic DNA was purified from petal tissues using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for genomic walk were designed from the 'Old Blush' EST sequences. Genomic walk was performed with the Universal Genome Walker TM 2.0 (Clontech, Mountain View, Ca, USA) according to the manufacturer's instructions. The resulting PCR products were cloned into the pGEMT-Easy vector (Promega, Madison, USA) and sequenced by Eurofins MWG Operon (Ebersberg, Germany). The different clones resulting from genomic walk were contigued using the Geneious R10 software (http://www.geneious.com). Primers (Supplementary Table 1) encompassing the whole coding region were defined on the assembly and PCR was performed on 'Old Blush' cDNA in order to clone the corresponding full-length cDNA into the pGEMT-Easy vector.

2.3. Expression of recombinant TPS in *Escherichia coli*

The coding sequence of linalool or linalool/nerolidol synthase was amplified using primers reported in Supplementary Table 1. For *RcLIN-NERS2* and *RcLINS*, the amplified sequence excluded the plastidial transit peptide. The PCR products were first cloned in the pENTR/D-TOPO entry vector (Invitrogen, USA), and subsequently transferred into the pHGWA (*RcLIN-NERS1*) or pHXGWA (*RcLIN-NERS2* and *RcLINS*) vectors of the pHGWA-based series (Busso et al., 2005) using the Gateway LR cloning reaction. *Escherichia coli* Rosetta (DE3) pLysS cells (Novagen, Darmstadt, Germany) were transformed with the recombinant expression vector by heat shock. The production of the heterologous protein was performed during an overnight incubation at 17 °C and 150 rpm in terrific broth

supplemented with 0.5% glycerol, 250 mM D-sorbitol and 2.5 mM betaine after induction with 0.5 mM IPTG. The cells were recovered by centrifugation and disrupted by a 30-min incubation in native lysis/binding buffer (50 mM NaH_2PO_4 (pH 8), 0.5 M NaCl, 20 mM imidazole, 5% glycerol, 0.5 mg.ml-1 lysozyme) followed by sonication. After lysate clarification by centrifugation, the recombinant protein was purified by binding to the Talon® metal affinity resin (BD Biosciences Clontech, Palo Alto, USA) according to the manufacturer's instructions. The 5' primer used to clone genes in the pENTR/D-TOPO entry vector comprised an additional thrombin recognition sequence for the hydrolysis of the His-tag/TPS (pHGWA) or thioredoxine/TPS (pHXGWA) recombinant protein by thrombin. The resin-bound protein was incubated overnight at 4 °C in 200 µl of 25 mM Tris-HCl (pH 7.5), 5% glycerol, 10 mM $MgCl_2$, 1 mM $MnCl_2$ supplemented with 10 units of thrombin (GE Healthcare). The following day the released TPS was recovered from the mixture by filtration. Protein concentration was measured using the Bio-Rad protein assay dye reagent (Bio-Rad, München, Germany) with bovine serum albumin as standard.

2.4. Enzymatic assays of recombinant protein activity

Twenty to fifty micrograms of purified recombinant protein were suspended in 490 µl of 25 mM Tris-HCl, (pH 7.5), 5% glycerol, 1 mM DTT, 1 mg.ml-1 BSA supplemented with 10 mM $MgCl_2$ and 1 mM $MnCl_2$ cofactors. The reaction was started by the addition of 10 µl geranyl or farnesyl diphosphate (Sigma) (final concentration 60 µM) and the mixture was overlaid with 500 µl of hexane. After a 3-hour incubation at 30 °C, the mixture was vigorously mixed and the upper hexane phase was collected, concentrated under a nitrogen stream and analyzed by GC-MS.

2.5. In planta activity of RcLINS

The *RcLINS* full length coding sequence was amplified using primers reported in Supplementary Table 1. Following cloning in the pENTR/D-TOPO entry vector, the sequence was transferred into the expression vector pMDC32 under the control of a double 35S promoter (Curtis and Grossniklaus, 2003). This construct was transformed into the *Agrobacterium tumefaciens* strain C58 (pMP90). Agrobacteria cultures expressing RcLINS were coinfiltrated in *Nicotiana benthamiana* leaves according to Batoko et al. (2000) together with agrobacteria expressing the P19 viral suppressor of silencing (Voinnet et al., 2003). After five days, infiltrated leaf sectors were cut into pieces and terpene extraction was performed as reported in the next paragraph.

2.6. Terpene extraction from tissues

Rose sepals, petals, stamens and leaves at different stages of development as well as infiltrated leaf sectors of transgenic tobacco plants were cut into fragments and extracted overnight at 4 °C in hexane containing 10 mg.l-1 camphor as an internal standard. The hexane/tissue ratio was 2 ml hexane per gram of fresh weight. The organic hexane phase was recovered. For extracts corresponding to transgenic tobacco leaves, the hexane phase was concentrated under a nitrogen stream before GC-MS analysis.

For the analysis of linalool or nerolidol glycosides, 1.5 g of ground petal material was resuspended in 4 ml 0.1 M citric acid, 0.2 M $Na_2HPO_4 12H_2O$ (pH 4.5) supplemented or not with 70 mg.ml-1 Rapidase AR2000 (DSM Food Specialties, Delft, Netherlands). The mixture was overlaid by 3 ml hexane with 10 mg.l-1 camphor in order to capture volatilized products. A four-hour incubation was performed at 37 °C under moderate agitation in order to avoid mixing the reaction and the overlaying hexane phase. The tube was finally vigorously vortexed to recover the released terpenes into the hexane phase, which was finally analyzed by GC-MS. The amount of linalool or nerolidol measured in the control without Rapidase

AR2000 was subtracted to the amount in the assay (presence of Rapidase AR2000) to yield the quantity of glycosylated product.

2.7. GC-MS analysis

For volatile product identification and quantification after enzymatic assays and in rose tissue extracts, GC-MS were performed on an Agilent GC 6850 gas chromatograph coupled with an Agilent 5973 ion trap mass detector. The instrument was equipped with a 30 m x 0.25 mm Agilent apolar capillary DB5 column. Helium was used as the carrier gas at a flow rate of 1ml.min-1. Temperatures of injector and detector were 250 °C. Injection volume was 2 µl in split (1:2) mode. Oven temperature settings were: 4 min at 60 °C after injection followed by a 4 °C.min-1 temperature ramp from 60 °C to 240 °C. Temperature was then kept on hold at 240 °C for 5 min. Molecule identification was performed using Wiley, NIST 05 and Adams mass spectra databases (Adams, 2007). The GC products' identifications were confirmed by comparing their retention times and mass spectra to those of authentic linalool and nerolidol samples (Payan Bertrand, France).

For linalool stereoisomer identification, gas chromatography was performed using an Agilent 6890 chromatograph equipped with a FID detector. The products were separated on a 30 m x 0,25 mm x 0,25 µm Agilent J&W CYCLOSIL-B chiral column. Helium was the carrier gas at a constant flow of 1.1 ml.min-1. Inlet and detector were set at 250 °C. Injection volume was 2 µl in pulsed splitless mode. Oven temperature was set at 60 °C for 5 min followed by a 1 °C.min-1 temperature ramp to 155 °C.

2.8. Assessment of terpene synthase expression by quantitative RT-PCR

Petals at stages 2, 3 and 4 were ground in liquid nitrogen and total RNA was extracted as described by Chang et al., 1993, with a ratio of 5 ml extraction buffer per gram of tissue. RNA were treated with the RQ1 RNAse-free DNAse (Promega, Madison, USA), quantified using a Nanodrop 2000C (Thermo Scientific, Wilmington, USA) and their quality was verified by electrophoresis on agarose gel. Five micrograms of total RNA were reverse transcribed at 50 °C for one hour with an oligo-dT primer using the SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA). Two independent reactions were performed for each RNA sample.

A fraction of petal cDNA issued from the *Rosa* x *hybrida* 'The McCartney rose' and 'Lady Hillingdon' cultivars was used for amplification of *RcLINS*, *RcLIN-NERS1* and *RcLIN-NERS2* using primers defined on 'Old Blush' sequences. The resulting PCR products were cloned into pGEMT-Easy and sequenced. Alignment of the homologous genes issued from the 'Old blush', 'The McCartney rose' and 'Lady Hillingdon' cultivars allowed to define fully conserved regions in which gene specific primers were designed for quantitative PCR (Supplementary Table 1).

Quantitative PCR was performed with CFX96™ real-time detection system (Bio-Rad, Hercules, USA) using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Hercules, USA). All reactions were carried out according to the manufacturer's protocol in a 20 µl volume using 2 µl of a 1/125 dilution of the reverse transcribed cDNA as template and 500 nM of each primer. Each cDNA sample was amplified twice with each primer combination in two independent runs. Since the RNA samples were reverse-transcribed in duplicate, this yielded four technical replicates per original sample. The PCR reactions were performed according to the same thermal profile: 95 °C for 30 s, followed by 40 quantification cycles (95 °C for 5 s, 60 °C for 30 s). At the end of the amplification, a melting-curve analysis (65 °C to 95 °C, one fluorescence read every 0.5 °C) allowed to check the specificity of the amplification.

Normalized expression values ($2^{-\Delta\Delta Cq}$ method, Livak and Schmittgen, 2001) were calculated by the CFX96TM data manager (Bio-Rad, Hercules, USA) using *EF1-α* and *α-TUBULIN* as reference genes (Supplementary Table 1).

2.9. Subcellular localization of RcLINS in tobacco plants

The *RcLINS* full-length coding sequence amplified using primers reported in Supplementary Table 1 was cloned in the expression vector pMDC83 (Curtis and Grossniklaus, 2003) to fuse RcLINS at the N-terminal of GFP. This construct was infiltrated in leaves of *Nicotiana benthamiana* as reported above. After five days, infiltrated leaf sectors were observed under a confocal microscope as reported in Magnard et al. (2015).

3. Results

3.1. Cloning of *Rosa chinensis* monoterpene synthases

Screening of a *Rosa chinensis* 'Old Blush' EST library (Dubois et al., 2012) using the browser https://lipm-browsers.toulouse.inra.fr/plants/R.chinensis for ESTs presenting homologies to known monoterpene synthases yielded very few sequences. In addition, homologies between these sequences indicated that these ESTs corresponded to only three different genes identified as *RcLINS*, *RcLIN-NERS1* and *RcLIN-NERS2*. All three of them were expressed at low level. Indeed, in the cDNA samples issued from the different flower stages used to build the EST database, the Reads Per Kilobase per Million mapped reads (RPKM) values were respectively 150 ("open flower" sample), 70 ("young closed flower" sample) and 60 ("young flower bud" sample) for *RcLINS*, *RcLIN-NERS1* and *RcLIN-NERS2*. For comparison purposes, RPKM values of 36034 and 4256 were found in "open flower" samples for respectively *RhNUDX1* and the sesquiterpene *Germacrene D synthase* (Guterman et al., 2002). In addition, to the opposite of *RhNUDX1* and *Germacrene D synthase*, which were only expressed in the "open" and "senescent flower" samples, *RcLINS*, *RcLIN-NERS1* and *RcLIN-NERS2* presented a larger expression profile as transcripts were detected in various non floral samples such as leaves or vegetative buds. However, expression levels in these samples were in the range of those detected in floral samples.

Since the recovered ESTs did not cover the whole TPS open reading frames, genomic walks were performed to complete the available sequences. RT-PCR was subsequently performed on 'Old Blush' flower cDNA with primers encompassing the whole coding regions in order to clone the corresponding full-length cDNA. The intron/exon profile was predicted following alignment of cDNA and genomic DNA. In addition, Feng et al. (2014) previously reported the cloning of a *Rosa rugosa* TPS sequence presenting homology to previously characterized linalool synthases. Since no enzymatic activity was done in Feng et al. (2014), isolation of the corresponding 'Old Blush' sequence was performed by PCR amplification on genomic DNA using primers designed on the *Rosa rugosa* sequence. The recovered fragment was extended by genomic walk. This fourth 'Old Blush' sequence was identified as *RcLIN-NERS3*. This gene is probably expressed at very low level in 'Old Blush' since no corresponding EST sequences could be identified in our database. However, since our blast analysis indicated that *RcLIN-NERS3* presented 91% identity to the *Fragaria vesca* nerolidol synthase 1 cDNA (XM_011462060.1, Aharoni et al., 2004), we used the available *FvNES1* cDNA to predict its intron/exon profile.

Figure 1 presents an alignment of the four protein sequences. The different motifs already reported to be implicated in catalysis by TPS: RRx8W, LQLYEASFLL, RDR, DDxxD and NSE/DTE (Degenhardt et al., 2009; Martin et al., 2010) are boxed on figure 1. Except for the RRx8W element, which is present only in RcLINS, all these motifs are well conserved. The RRx8W motif however is commonly absent in TPS producing acyclic

compounds such as linalool or geranyl-linalool, especially enzymes of the TPS-g subfamily (Degenhardt et al., 2009; Martin et al., 2010). The modification of the RDR motif to RDQ seen in RcLIN-NERS1, 2 and 3 has also already been reported for PtTPS3 and PtTPS15, two poplar linalool/nerolidol synthases (Irmisch et al., 2014).

With its 7 exons, RcLINS {GenBank accession numbers: MG673509 (cDNA) and MG673516 (genomic DNA)} presents the canonical structure of angiosperm-specific TPS belonging to the TPS-a, TPS-b and TPS-g subfamilies (Martin et al., 2010; Chen et al., 2011). In addition, the algorithms ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/), Predotar 1.04 (https://urgi.versailles.inra.fr/Tools/Predotar), SCLpredT (http://distillf.ucd.ie/distill/) and WoLF PSORT (https://www.genscript.com/wolf-psort.html) all clearly predicted a plastidial transit peptide, corresponding to the first 45 amino acids. Compared to RcLINS, RcLIN-NERS1 {GenBank accession numbers: MG673510 (cDNA) and MG673513 (genomic DNA)} was shorter in the 5' region, the putative methionine initiation codon being located at the end of exon one. A stop codon was present 6 bp upstream of this methionine. In addition, a putative TATA box sequence (TATAAA) is located 95 bp ahead of the initiation methionine, with no alternative start codon between this box and the presumed initiation codon. We then assumed that the RcLIN-NERS1 sequence presented in figure 1 corresponded to a protein that can genuinely be translated in vivo. The different algorithms agreed on the absence of a plastidial transit peptide, which was not surprising considering the shorter 5' region compared to RcLINS.

Cloning of *RcLIN-NERS2* DNA by genomic walk yielded four sequences, which presented from 93,8 to 96,4% identity between them at the protein level (Supplementary Figure 1). They also show strong (83,3 to 84,3% depending on the sequence) identity to the *Fragaria ananassa* FaNES1 and FaNES2 linalool/nerolidol synthases (Aharoni et al., 2004). Since the 'Old Blush' cultivar is diploid, these four clones presumably correspond to two allelic versions of two very similar genes, eventually issued from a duplication event. Three of these sequences harbored a stop codon just 42 bp after the initiation codon corresponding to the initiation codon of the *FaNES2* gene (Supplementary Figure 1). However, an alternative initiation codon present 213 bp downstream of the first ATG at the end of exon 1 could yield a 509 amino acid protein. In the fourth sequence, presented on Figure 1 {GenBank accession numbers: MG673511 (cDNA) and MG673514 (genomic DNA)}, the stop codon was replaced by a glutamine allowing the production of a longer 580 amino acid protein. The ChloroP software predicted a putative plastidial transit peptide corresponding to the first 31 amino acids, which was confirmed by the Predotar, SCLpredT and WoLF PSORT programs. However, this peptide would not be present in case the alternative start codon was used for protein production. To the opposite of most TPS belonging to the TPS-a, -b and -g subfamilies, the alignment of *RcLIN-NERS2* genomic and cDNA sequences showed that this gene was devoid of the sixth intron as was already reported for *FaNES1* and *FaNES2* (Aharoni et al., 2004).

The last sequence (RcLIN-NERS3, GenBank accession number: MG673515) is homologous to the putative *Rosa rugosa* linalool synthase described by Feng et al. (2014) (accession JN675703) and *Fragaria vesca* Nerolidol synthase 1 (FvNES1: XM_011462060.1, (Aharoni et al., 2004). The sequence of the original *Rosa rugosa* protein reported by Feng et al. (2014) started at the beginning of exon 3. Genomic walk was then performed on *Rosa chinensis* 'Old Blush' in order to extend the sequence on the 5' side and to assess if the gene was complete in this species. Unfortunately, the recovered sequences presented no homology to *FvNes1* and included numerous stop codons. Consequently, this rose protein is a truncated version of a protein present in other members of the Rosaceae family and is presumably devoid of TPS activity. The lack of ESTs corresponding to this gene in our 'Old Blush' database confirms the assumption that this gene is actually a pseudo-gene. The available

RcLIN-NERS3 sequence showed a close proximity to RcLIN-NERS2 (90,8 % identity at the protein level) and similarly to *RcLIN-NERS2*, *RcLIN-NERS3* is devoid of the sixth intron.

3.2. Enzymatic activity of recombinant rose monoterpene synthases

RcLINS, RcLIN-NERS1 and RcLIN-NERS2 coding sequences were used for production of recombinant proteins in the *E. coli* Rosetta strain. Since the presence of a plastidial transit peptide can lead to the production of an insoluble protein, it was removed from the RcLINS sequence. The coding sequence of RcLIN-NERS2 was conserved starting from the second alternative start codon (see above). Due to its important deletion on the 5' side, RcLIN-NERS3 was supposed devoid of activity and therefore was not included in this survey. Activities of the purified recombinant proteins were assessed on the GPP and FPP substrates, and the products analyzed by GC-MS (Figure 2).

All three proteins proved to be active on GPP and produced the monoterpene linalool as unique (RcLINS and RcLIN-NERS2) or main (RcLIN-NERS1) product, as assessed by comparison of retention time and mass spectra with those of a linalool standard. However, chiral analysis of the stereoisomers produced by the different enzymes showed that RcLINS produced exclusively the (-)-(*3R*) isomer of linalool to the opposite of RcLIN-NERS1 and 2 which only produced (+)-(*3S*)-linalool. Minor amounts of other monoterpenes (*ß*-myrcene, limonene and *Z* and *E*-ocimene) were also produced by RcLIN-NERS1. Only RcLIN-NERS1 and RcLIN-NERS2 proved to be able to catalyse FPP transformation into sesquiterpenes and in both cases, the sole resulting product was nerolidol. Consequently, both RcLIN-NERS1 and RcLIN-NERS2 were identified as bifunctional linalool nerolidol synthases. Conversely, RcLINS is an exclusive linalool synthase.

3.3. Relatedness of rose enzymes to other linalool, nerolidol and geranyllinalool synthases

A neighbor joining tree including RcLINS, RcLIN-NERS1 and RcLIN-NERS2 together with TPS whose linalool, nerolidol and/or geranyllinalool synthase activities have been reported in the literature is presented in Figure 3. Enzymes presenting these catalytic properties can be found in the TPS-a, b, d, e/f, and g subfamilies of terpene synthases with a prevalence in the b and g groups. A characteristic feature of the sequences belonging to the a, b and d subfamilies is the presence of the canonical RRx8W motif, absent only in *Coffea arabica* CCM43928.1. The enzymes of the TPSg and e/f subfamily are all devoid of this motif.

Both RcLIN-NERS1 and RcLIN-NERS2 linalool/nerolidol synthases cluster within the TPS-g. When both the GPP and FPP substrates were tested, most of the enzymes of this group were reported to be able to catalyze both linalool and nerolidol synthesis, the only exception being the *Citrus unshiu* linalool synthase (BAP75559.1). Although some of these enzymes are also able to produce geranyllinalool from GGPP (*Vitis vinifera* ADR74213.1, *Medicago truncatula* AAV36466.1, *Zea mays* AAX99149.1), others are devoid of this ability (*Populus trichocarpa* AEI52903.1, *Solanum lycopersicum* AEP82769.1, *Oryza sativa* ACF05530.1). When a chiral analysis of the products was performed, these enzymes produced the (+)-(*3S*) linalool or nerolidol stereoisomers, the only exception being *Ocimum basilicum* AY693647.1.

By contrast, RcLINS clusters in the TPS-b subgroup. Similarly to RcLINS, most enzymes of this subfamily are strict linalool synthases and are unable to catalyse nerolidol production from FPP. Only three of them (*Populus trichocarpa* AII32476.1 and AEI52904.1 and *Hedychium coronarium* AGY49283.1) produce sesquiterpenes, which however are generally not nerolidol. None of the members of this group has been proven to be able to catalyse geranyllinalool synthesis. Most of these enzymes produce the (-)-(*3R*)-linalool isomer, except for *Coriandrum sativum* AHC54051.1 and *Populus trichocarpa* AII32476.1 yielding respectively (+)-(*3S*)-linalool and a mix of both isomers.

The TPSe/f subfamily regroups enzymes with a dual nerolidol/geranyllinalool synthase activity (*Vitis vinifera* ADR74220.1, *Solanum lycopersicum* AIL54748.1, *Tripterygium wilfordii* AQA26340.1) or which are strict geranyllinalool synthases (*Populus trichocarpa* AII32474.1, *Arabidopsis thaliana* AAN71959.1, *Nicotiana attenuata* AIL54746.1). The only enzyme with a proven linalool synthase activity is *Clarkia breweri* AAC49395.1, however, its activity has not been tested on FPP and GGPP. As expected from a gymnosperm terpene synthase, *Picea sitchensis* ADZ45501.1 clusters in the TPS-d subgroup. This enzyme is a strict (-)-(*3R*)-linalool synthase devoid of activity on FPP and GGPP. Finally, a single enzyme was found to cluster in the TPS-a subfamily, namely *Citrus unshiu* BAP75561.1, which presents a dual linalool/nerolidol synthase activity.

3.4. Linalool production and expression of TPS in roses

Some of our previous experiments have already reported linalool production in some rose cultivars. Three of these cultivars were chosen for extensive linalool and nerolidol quantification in different tissues at four stages of development by hexane extraction and GC-MS. *Rosa chinensis* 'Old Blush' was chosen since the three linalool or linalool nerolidol synthases have been cloned in this cultivar. In addition, 'Old Blush' is characterized by the production of geraniol and derivatives through the Nudix pathway (Figure 4). *Rosa* x *hybrida* 'Lady Hillingdon' was selected due to the absence of accumulation of significant quantities of geraniol and derivatives prior to stage 4 of flower development (Figure 4). Consequently, it could be assumed that prior to this stage, linalool synthases are not in competition with RhNUDX1 for the GPP substrate. On the contrary, *Rosa* x *hybrida* 'The McCartney rose' already produces abundant monoterpenes through the Nudix pathway at stage 3 of flower development (Figure 4).

No nerolidol accumulation was detected in any of the samples included in our study. The dimethyl nonatriene homoterpene, which results from nerolidol cleavage (Richter et al., 2016), was also absent in the different extracts. On the contrary, linalool was detected in 'Lady Hillingdon' flowers from stages 1 to 5 (Figure 5B). In sepals, the amount was greater at early stages and decreased throughout flower development. This decrease correlated with an increase in petals, in which linalool abundance peaked at stage 3. This maximal amount of linalool (34,3 +/- 1,5 ng.mg-1 dry weight in stage 3 petals) was in the range of the quantity of geraniol and derivatives, which appear in noticeable quantities in stage 4 petals (65 +/- 34,8 ng.mg-1 dry weight). Linalool accumulation under a non-volatile glycosylated form was reported in different plants such as *Vitis vinifera* and *Osmanthus fragrans* (Martin et al., 2012; Zeng et al., 2015) as well as in transgenic petunia and arabidopsis expressing linalool synthases (Lücker et al., 2001; Aharoni et al., 2003). Glycosylation of linalool could be a way for the cell to cope with the potential toxicity of this compound (Lücker et al., 2001). Since glycosylated forms cannot be readily detected by GC-MS, samples of petals at stages 2, 3 and 4 were submitted to deglycosylation prior to GC-MS analysis. However, only low amounts of glycosylated linalool could be detected, corresponding roughly to one tenth of the amount of free linalool (data not shown). Finally, the amount of linalool present in leaves and stamens was low compared to sepals and petals.

'Old Blush' tissues were characterized by very weak quantities of linalool which was mainly detected at stage 4 (Figure 5B). No linalool was stored in glycosylated form. Linalool levels in some of 'The McCartney rose' samples were similar to those detected in 'Lady Hillingdon'. However, the range of tissues/stages accumulating this compound was restricted compared to what was detected in 'Lady Hillingdon'. A noticeable difference was the important quantity detected in stamens of 'The McCartney rose' stage 4 flowers. Similarly to 'Lady Hillingdon', deglycosylation allowed the detection of some more linalool, mainly at stage 3, but the recovered quantities were lower than the quantities of free linalool (28% and

10% at respectively stages 3 and 4; data not shown). To the opposite of what was seen for 'Lady Hillingdon', petal samples of 'The McCartney rose' were rich in geraniol and derivatives: at stage 4, the amount of monoterpenes derived from the Nudix pathway (1857 +/- 119.8 ng.mg-1) is roughly 82 times higher compared to the quantity of free linalool (22.7 +/- 6.1 ng.mg-1). (For a comparison of the respective linalool and geraniol plus derivatives levels in 'The McCartney rose' petals, see also Figure 4.)

Finally, the analysis of the linalool stereoisomers present in rose flowers was performed with samples presenting the maximum accumulation of this compound, namely 'Lady Hillingdon' stage 3 and 'The McCartney rose' stage 4 petal and stamen samples. Only (-)-(*3R*)-linalool was detected in the three samples (Figure 5C).

Overall, our results show that linalool accumulation is generally higher in petal samples. The expression of rose linalool and linalool/nerolidol synthases was consequently evaluated during petal development using quantitative RT-PCR (Figure 6). All three enzymes were expressed in these samples, albeit at different levels depending on the cultivar and stage of development. *RcLINS* and *RcLIN-NERS2* presented somewhat similar expression profiles: they were expressed in the three cultivars, although their expressions were lower in 'The McCartney rose'. Both enzymes showed a peak in expression at stage 3. However, in 'Lady Hillingdon', the extent of *RcLIN-NERS2* expression was reduced compared to *RcLINS*. Compared to *RcLINS* and *RcLIN-NERS2*, *RcLIN-NERS1* was expressed earlier during petal development (higher expression at stage 2) and was virtually absent in the 'The McCartney rose' cultivar.

3.5. RcLINS in planta activity

RcLINS is the only enzyme characterized in this study which is able to produce in vitro the (-)-(*3R*)-linalool isomer found in rose flowers. Its capacity to synthesize linalool in planta was consequently verified following transient transformation in *Nicotiana benthamiana* leaves (Figure 7). Due to the complexity of the tobacco leaves' GC profile, the mass spectrum was recovered in SIM (selected ion monitoring) mode to selectively record ion m/z 93, characteristic of linalool. This ion was selectively present in RcLINS transformed tobaccos and absent from control plants, confirming RcLINS involvement in linalool production in planta.

3.6. Subcellular localization of RcLINS

The RcLINS coding sequence was finally fused to GFP in the pMDC83 expression vector and transformed into *Nicotiana benthamiana* leaves to assess subcellular localization of this protein (Figure 8). Similar experiments were not performed with RcLIN-NERS1 and RcLIN-NERS2 since only RcLINS is likely to produce the (-)-(*3R*)-linalool present at detectable levels in rose flowers. Plastids were revealed by autofluorescence of the chlorophyll. RcLINS localization was detected as a punctuated pattern at the surface of plastids (Figure 8A, B) and was different from that of the SsLPPS *Salvia sclarea* diterpene synthase (Caniard et al., 2012) used as a control for localization in the stroma of the plastid (Figure 8C). However, it was difficult to assess whether RcLINS was localized inside a sub-compartment of the plastid close to plastid membrane or directly inserted into the plastid membrane. It could also be located in a cellular compartment external to the plastid but which remains in close contact with the plastid membrane.

4. Discussion

4.1. Several enzymes with linalool or linalool nerolidol synthase activities are present in the rose's genome.

The rose's genome contains at least four genes coding for linalool or linalool/nerolidol synthases (Table 1). One of these genes is a pseudogene lacking a significant part of the 5' sequence, two encode bifunctional (+) linalool nerolidol synthases and one a monofunctional (-) linalool synthase. The number of different genomic clones related to *RcLIN-NERS2* suggests a duplication of this gene and the sequence homology with *RcLIN-NERS3* suggests that this latter gene also results from a duplication of *RcLIN-NERS2* followed by extensive deletion in the 5' region. Rose genome sequencing, which is currently in progress, will likely allow us to confirm these hypotheses by verifying the spatial proximity of these sequences on the chromosomes.

According to Landmann et al. (2007) and Degenhardt et al. (2009), the formation of the acyclic monoterpene linalool is a simple mechanism compared to the synthesis of most other monoterpenes, especially cyclic products: after initial ionization of geranyl diphosphate, the geranyl cation undergoes a simple allylic rearrangement and is rapidly quenched by a water molecule. A similarly simple mechanism can be proposed for nerolidol and geranyllinalool formation from respectively farnesyl diphosphate and geranylgeranyl diphosphate: carbocation formation is followed by allylic rearrangement and water quenching (Tholl et al., 2011). Linalool or nerolidol synthases can thus be viewed as defective forms of TPS that may only catalyze the ionization of the substrate and whose active site allows water access to the carbocation. Consequently, any mutation preventing a mono or sesquiterpene synthase to either stabilize the substrate in the carbocation state or extensively rearrange this carbocation molecule would lead to the appearance of such an enzyme. The occurrence of linalool synthases in the different TPS subfamilies confirms this assumption (Landmann et al., 2007). In the *Mentha spicata* limonene synthase, the exchange of a charge stabilizing tryptophan residue by a nonaromatic residue caused the formation of products characteristic of a premature termination of the reaction, including linalool (Srividya et al., 2015). In line with this model, the RcLINS and RcLIN-NERS1/2 rose linalool and linalool/nerolidol synthases correspond to independent evolutive schemes and the ability to catalyze linalool or nerolidol production seems to have appeared independently several times in this species.

Furthermore, linalool or linalool/nerolidol synthase activities are well correlated with their belonging to one of the subfamilies: RcLINS clusters with other mono-functional linalool synthases into the TPS-b subfamily. Similarly to RcLINS, most of the b class enzymes with linalool synthase activity have been reported to produce the (*3R*)-(-) isomer. Besides linalool synthases, different monoterpene synthases and isoprene synthases can be found in the TPS-b subfamily (Chen et al., 2011). As for the bi-functional RcLIN-NERS1 and RcLIN-NERS2, they belong to the TPS-g subgroup together with other bi-functional linalool/nerolidol or trifunctional linalool/nerolidol/geranyllinalool synthases. The linalool isomer produced by synthases of this group is usually of the (*3S*)-(+) type as it is for RcLIN-NERS1 and 2. This confirms that (*3S*)-(+) and (*3R*)-(-) linalool synthases do not cluster closely with each other (Raguso, 2016). On a more general point of view, the TPS-g clade is quite heterogeneous and contains mono-, sesqui- or diterpene synthases. However, one characteristic of these enzymes is the prevalence among their products of acyclic compounds such as linalool or nerolidol (Martin et al., 2010; Chen et al., 2011).

4.2. The weak expression of the characterized TPS explains the low abundance of linalool in roses.

In vitro enzymatic activity has brought clear information on the potential activity of these TPS. However, the question of understanding their actual in vivo function in rose flowers arises. The analysis of 'The McCartney rose' and 'Lady Hillingdon' petal and stamen extracts by chiral GC-MS shows that these rose cultivars only produce the (-)-(*3R*)-linalool isomer. On the other hand, RcLINS is the only characterized enzyme, which is able to

produce this isomer. Consequently, we can assume that the linalool produced in rose flowers derives from the activity of this single terpene synthase. As can be expected for a monoterpene synthase, RcLINS is targeted towards the plastidial compartment. However, our study showed that RcLINS is not present in the stroma but is localized in seemingly vesicular structures which seem to be in close association with the plastid surface. A similar localization has already been reported for several Arabidopsis enzymes involved in terpene metabolism: the deoxyxylulose 5-phosphate synthase (DXS), the deoxyxylulose 5-phosphate reductoisomerase (DXR) (Pulido et al., 2013; Perello et al., 2016), the TPS10 and TPS14 linalool synthases (Ginglinger et al., 2013) and four geranylfarnesyl diphosphate synthases (Nagel et al., 2015). The reason for this particular localization is not clear. In the case of DXS, Perello et al. (2016) propose that this punctuate pattern may result from the aggregation of misfolded inactive proteins to the plastidial membrane. The explanation would be different for DXR, which is less prone than DXS to instability. In that case, the excess production of the protein could lead to its capture into intra-plastidial vesicles that could eventually be released into the cytosol for degradation (Perello et al., 2016). The transcription of RcLINS from a 35S promoter in transgenic plants could conceivably lead to such overexpression of the protein. However, in the case of Arabidopsis TPS10 and 14, this particular localization is supposed to promote direct product transfer from the linalool synthases to two cytochrome P450s localized on endoplasmic reticulum regions which are in direct contact with the plastid envelope. The P450 enzymes have indeed been shown to be able to metabolize linalool into different oxidized compounds which can even be further modified by glycosylation (Ginglinger et al., 2013). A similar channeling process between RcLINS and yet unknown enzymes could be postulated, although no linalool oxides could be detected in our GC-MS analysis and no substantial amount of glycosylated linalool was present in flower samples. However, the low RcLINS expression level may prevent linalool production in sufficient amount for further transformation. The localization of RcLINS at the interface between cytosol and plastid would also conceivably place this enzyme in a strategic location allowing access to two potential GPP substrate pools: GPP produced through the MEP pathway inside the plastid and the postulated GPP pool present in cytosol which is used by the cytosolic RhNUDX1 enzyme (Magnard et al., 2015).

Finally, an attempt to link linalool production to RcLINS expression shows a good correlation between those two parameters in ‘Lady Hillingdon’, with a maximum in mRNA production occurring shortly before linalool accumulation. Consequently, linalool synthesis is probably regulated at the transcriptional level in this cultivar. However, this is obviously not the case in ‘Old Blush’, in which linalool production was extremely low despite RcLINS transcription levels similar to those detected in ‘Lady Hillingdon’. The opposite is seen in the ‘The McCartney rose’ flowers, which are characterized by a weaker RcLINS transcription compared to ‘Old Blush’, together with a linalool production in the range of that seen in ‘Lady Hillingdon’. Hypotheses to explain this situation include post-transcriptional repression or low availability of substrate in ‘Old Blush’ and an enhanced stability of the transcript or protein in the ‘The McCartney rose’. However, in this latter cultivar, the transcription of a yet unknown linalool synthase which would not be detected in the ‘Old Blush’ ESTs cannot be excluded.

The analysis of genomic clones corresponding to RcLIN-NERS2 showed at least four strongly homologous sequences. Three of them possess a Stop codon at the beginning of the gene, followed by a putative alternative Start codon. This situation is reminiscent of what has been described in *Fragaria ananassa* for the possible orthologs of RcLIN-NERS2: the closely related FaNES1 (P0CV94.1) and FaNES2 (P0CV95.1) (Aharoni et al., 2004). Indeed, although FaNES2 presents a targeting peptide, FaNES1 shows a stop codon just downstream of the initiation methionine. However, the use of an alternative methionine yields the

production of a functional cytosolic protein. Moreover, the plastidial FaNES2 protein is presumably not very important for the production of the linalool and nerolidol detected in the strawberry fruit, since FaNES1 presents a much higher expression (Aharoni et al., 2004). A somewhat similar situation may be found with the *Rosa* RcLIN-NERS2 sequences: sequencing of *RcLIN-NERS2* cDNA clones issued from different species and cultivars showed that in all cases they encoded the shorter form. This raises questions on the effective in vivo translation of a plastidial full-length form of this protein similarly to what is seen for the *Fragaria* genes. In case most of the RcLIN-NERS2 protein should be cytosolic, it would join RcLIN-NERS1 in this compartment, since RcLIN-NERS1 is devoid of plastidial transit peptide. In the cytosol, these enzymes should encounter the FPP substrate produced through the mevalonate pathway. In roses, a substantial amount of GPP should also be present in this compartment to provision RhNUDX1. However, the absence of nerolidol and of its derivative dimethyl nonatriene, together with the absence of (+)-linalool in the rose flower extracts, suggests a lack of in vivo RcLIN-NERS1 and RcLIN-NERS2 activity.

This comparison shows that roses and strawberries, two members of the Rosaceae family, have evolved different enzymes to produce linalool. *Fragaria ananassa* uses the (+)-(*3S*)-linalool nerolidol FaNES1 synthase (Aharoni et al., 2004), whereas the orthologous RcLIN-NERS2 is ineffective in rose flowers. On the contrary, the small quantity of linalool produced in rose flowers derives from the activity of the RcLINS (-)-(*3R*)-linalool synthase which is unable to produce nerolidol. An enzyme showing extensive identity (78%) to RcLINS is present in the *Fragaria vesca* genome (XP004289147.2); however, to our knowledge, its implication in linalool production has never been reported. Although both (-)-(*3R*) and (+)-(*3S*)-linalool are involved in pollination and defense, the two stereoisomers differ in their respective implications in these processes (Raguso, 2016). Yet, linalool has never been linked to ecological processes in roses so far.

The main hypothesis to explain the absence of RcLIN-NERS1 and RcLIN-NERS2 activity together with the low production of (-)-(*3R*)-linalool in the characterized rose cultivars is likely due to the low expression levels of *RcLIN-NERS1*, *RcLIN-NERS2* and *RcLINS*. Indeed, the comparison of the abundance of ESTs in the 'Old Blush' database shows that RPKM values for *RcLINS*, *RcLIN-NERS1* and *2* in samples corresponding to flower stages of development are 240 to 600 times lower than those found for *RhNUDX1*. Similarly, a comparison involving only terpene synthases {ie *RcLINS*, *RcLIN-NERS1* and *2* against germacrene D synthase (Guterman et al., 2002)} shows that germacrene D synthase is 30 to 70 times more expressed. These values can be materialized by an observation of the upper chromatogram on Figure 4: geraniol derivatives and germacrene D are abundant while linalool accumulation is not detectable in this 'Old Blush' petal sample at stage 3. In regards to these results, it is difficult to correlate TPS expression and linalool accumulation. Indeed, for both expression and linalool production, we are dealing with low quantities. Furthermore, variations in linalool production can be due to subtle modifications in gene expression, mRNA stability, effectiveness of translation, activities of the different TPS, respective affinity of linalool synthase and RhNUDX1 for GPP or the availability of this substrate in different compartments... Considering this last point, RcLINS, which is not significantly more expressed than RcLIN-NERS1 and 2, but is targeted to a different compartment, leads to the production of low but detectable amounts of linalool.

4.3. Two pathways of different importance for monoterpene production in roses?

Many rose cultivars (including 'Old Blush' and 'The McCartney rose') are characterized by the abundant production of acyclic monoterpenes: geraniol and derivatives (nerol, citronellol, geranial) through the Nudix pathway (Figure 4). Conversely, other monoterpenes produced through conventional monoterpene synthases (including linalool) are

much less abundant in roses. Yet, these two monoterpene biosynthesis pathways are most likely interconnected since both rely on a common precursor, namely GPP. Our original hypothesis assumed that the monoterpene synthase dependent compounds (including linalool) were synthesized at low levels due to the use of the GPP substrate by the enzymes of the Nudix pathway. However, the low amount of linalool and the absence of nerolidol in rose petals are more likely linked to the extremely low expression of the characterized terpene synthases compared to RhNUDX1. Even in 'Lady Hillingdon' petals, the presumed low activity of the Nudix pathway does not translate into a higher expression of the conventional linalool or linalool/nerolidol synthases (unless geranyl monophosphate actually produced by the NUDX protein cannot be transformed into geraniol due to the absence of a dedicated phosphatase).

It is thus possible that roses result from an evolutive scheme in which a decrease in the expression of monoterpene synthase genes (and possibly mutations in these genes) left room for the emergence of the Nudix pathway. Alternatively, a decrease in the importance of the monoterpene synthase family may have resulted from previous emergence of the Nudix pathway, which proved a more efficient way to produce monoterpenes due for instance to the higher affinity of RhNUDX1 for the GPP substrate or a more adequate subcellular localization: cytosolic (RhNUDX1) versus plastidial (RcLINS). The Nudix pathway of monoterpene biosynthesis has so far only been characterized in the *Rosa* genus. The question of its evolutive emergence and in particular of its presence in other members of the rosaceae family is still unresolved. In the case where it would be present in other genera of this family, the relative expression and activity of TPS and NUDX enzymes might be informative to understand the links between the two monoterpene biosynthesis pathways.

In strawberries, the terpenoid profile of the fruit of the cultivated strawberry (*Fragaria ananassa*) is dominated by linalool and nerolidol, whereas the wild strawberry (*Fragaria vesca*) produces other monoterpenes (pinene, myrcene, phellandrene…). These compounds are synthesized by highly expressed monoterpene synthases: the *Fragaria ananassa* FaNES1 linalool/nerolidol synthase, and the *Fragaria vesca* FvPINS pinene synthase (Aharoni et al., 2004). Consequently, the expression of linalool synthase likely shows a marked difference between the *Rosa* and *Fragaria* genera. According to our hypotheses above, the high expression of TPS should correlate with the absence of the Nudix pathway in strawberries. Since no geraniol or derivatives are present in strawberry fruit, it can be assumed that this pathway is not active in this organ. However, additional analyses of monoterpene synthases and NUDX expression in strawberry flowers are needed to understand the link between the two monoterpene biosynthesis pathways.

In addition to their weak expression, the number of terpene synthases described in roses is low: so far only the linalool and linalool/nerolidol synthases characterized here and one germacrene D synthase (Guterman et al., 2002) have been reported. The RcLIN-NERS3 sequence shows that pseudogenes are likely to be present in the roses' genome. In apple, another member of the rosaceae family, 55 TPS genes have been predicted throughout the genome. However only 10 (18%) of them are likely to encode functional enzymes, including three monoterpene synthases (Nieuwenhuizen et al., 2013). In that respect, apples are quite different from other plants such as arabidopsis, grapes or tomatoes in which respectively 32, 69 and 29 potentially functional TPS have been predicted following genome sequence analysis. These values correspond to 80%, 45% and 66% of the total number of TPS sequences present throughout these genomes (Aubourg et al., 2002; Martin et al., 2010; Falara et al., 2011). The sequencing of the rose genome, which is currently in progress will reveal the actual number of TPS genes. In case numerous pseudogenes should also exist in that species, this characteristic may result from the lower selection pressure applied to the TPS family due to the emergence of the Nudix pathway of monoterpene biosynthesis. In addition,

other rose monoterpene synthases can be cloned using cDNA issued from other cultivars or species (including botanical species) producing different monoterpenes (pinene, myrcene, limonene, ocimene…), although in most cases the level of production of these compounds will be low. The characterization and expression study of these enzymes will likely be informative regarding the relevance of the TPS family for monoterpene biosynthesis in roses.

Author contributions

Conception and design of the study: JLM, FJ, SB. Realization of the experiments: ARB, JLM, FB, FJ, AC, BB. Analysis of the data: JLM, ARB, FJ. Writing of the manuscript: JLM, SB, FJ.

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Figure legends

Figure 1: Alignment of *Rosa chinensis* linalool and linalool nerolidol synthases RcLINS, RcLIN-NERS1, 2 and 3. The portions of the sequence highlighted in black present 100% similarity; the portions highlighted in gray are 60 to 80% similar. The RcLINS and RcLIN-NERS2 predicted plastid targeting peptides are underlined with dashed lines. The characteristic TPS catalytic motifs (RRx8W, LQLYEASFLL, RDR, DDxxD and NSE/DTE, see text) are boxed. The positions of introns are indicated by vertical dashed lines; the sixth intron is present only in RcLINS and RcLIN-NERS1. In the RcLIN-NERS2 sequence, the first arrowhead points to a polymorphic position that corresponds to either a stop codon or a glutamine. The second arrowhead points to the alternative start codon.

Figure 2: GC-MS analysis of the volatile products synthesized by recombinant RcLINS, RcLIN-NERS1 and RcLIN-NERS2. After expression in *Escherichia coli* and purification, the enzymes were incubated with the GPP (left and right columns) and FPP (middle) substrates. Left and middle columns: GC-MS analysis of the resulting products on an apolar capillary DB5 column. Retention times and mass spectra were compared to those of a linalool standard or to those of a mix of nerolidol plus farnesol standards (bottom line, left and middle). Right column: enantioselective analysis (CYCLOSIL-B chiral column) of linalool produced by the three enzymes upon incubation with GPP. A mixture of the (+/-)-(*3S*/*3R*)-linalool stereoisomers was used as standard (bottom lane, right). 1, 2, 3: minor products resulting from RcLIN-NERS1 activity on GPP: ß-myrcene (1), limonene (2), (*Z*) and (*E*) ocimenes (3).

Figure 3: Relatedness of the cloned rose TPS proteins to other functionally characterized linalool, nerolidol, geranyllinalool synthases. Only the portion of the sequences corresponding to the conserved PlantTerpene Cyclase Class 1 domain (conserved domain cd 00684 from the NCBI Conserved Protein Domain Database) was used for the alignment. RcLINS and RcLIN-NERS1, 2 described in this paper are shown in bold. RcLIN-NERS3 whose sequence is very close to RcLIN-NERS2 was not included. The tree was built using the Geneious Tree Builder (Geneious R10 Biomatters Ltd.), with the Neighbor-Joining method. The Jukes-Cantor model was used for calculation of the genetic distances. The outgroup is the *Rosa chinensis* Germacrene D synthase (MG673512) orthologous to the *Rosa* x *hybrida* Germacrene D synthase (Guterman et al., 2002). Bootstrap values are shown next to each node. Clusters corresponding to the TPS-a, b, d, e/f and g subfamilies are indicated.

Figure 4: Representative GC-MS analysis of hexane-extracted products recovered from stage 3 petals from *Rosa chinensis* 'Old Blush' or *Rosa* x *hybrida* 'Lady Hillingdon' and 'The McCartney rose' (see Figure 5 for a definition of the stages). Only products corresponding to terpenes or derivatives are labeled: 1: linalool, 2: nerol, 3: neral, 4: geraniol, 5: geranial, 6: geranyl acetate, 7: ß carene (putative identification), 8: dihydro-ß-ionol, 9: germacrene D, 10: putative δcadinene, 11: putative elemol. Products 1 through 6 correspond to monoterpenes. In 'Lady Hillingdon', the product with the same retention time as geranial is 3-5-dimethoxytoluene. Products 2, 3, 5, 6 are supposed to be derived from 4 (geraniol).

Figure 5: In vivo linalool production in sepals, petals, stamens and leaves of *Rosa x hybrida* 'Lady Hillingdon' and 'The McCartney rose' and *Rosa chinensis* 'Old Blush' at different stages of development. A: Flower morphology corresponding to each of the five developmental stages (St.1 to 5). The buds and flowers are issued from 'The McCartney rose' cultivar. B: GC-MS analysis of hexane-extracted compounds present in the different samples was used to quantify linalool accumulation (ng.mg-1 dry weight). The values are the means

and standard deviations obtained from three independent samples. Black bars: sepals, white: petals, gray: stamens, hatched: leaves. C: chiral analysis of the linalool stereoisomers found in petals or stamens. From top to bottom: 'Lady Hillingdon' stage 3 petals; 'The McCartney rose' stage 3 petals; 'The McCartney rose' stage 3 stamens; mixture of (+/-)-(*3S*/*3R*)- linalool stereoisomers used as standard.

Figure 6: Transcriptional activity of *RcLINS*, *RcLIN-NERS1* and *RcLIN-NERS2* in petals of *Rosa* x *hybrida* cultivars 'Lady Hillingdon' and 'The McCartney rose' and *Rosa chinensis* 'Old Blush' at different stages of development measured by quantitative RT-PCR. The analyses were performed at stages 2 (black bars), 3 (white bars) and 4 (gray bars). Transcript levels of terpene synthases are normalized to those of *EF1-*α and α*-TUBULIN*. Values are the means and standard deviation of four technical replicates. Lower graph: Linalool accumulation in the same samples reported from Figure 5 (stages 1 and 5: hatched bars, stage 2: black, stage 3: white and stage 4: gray).

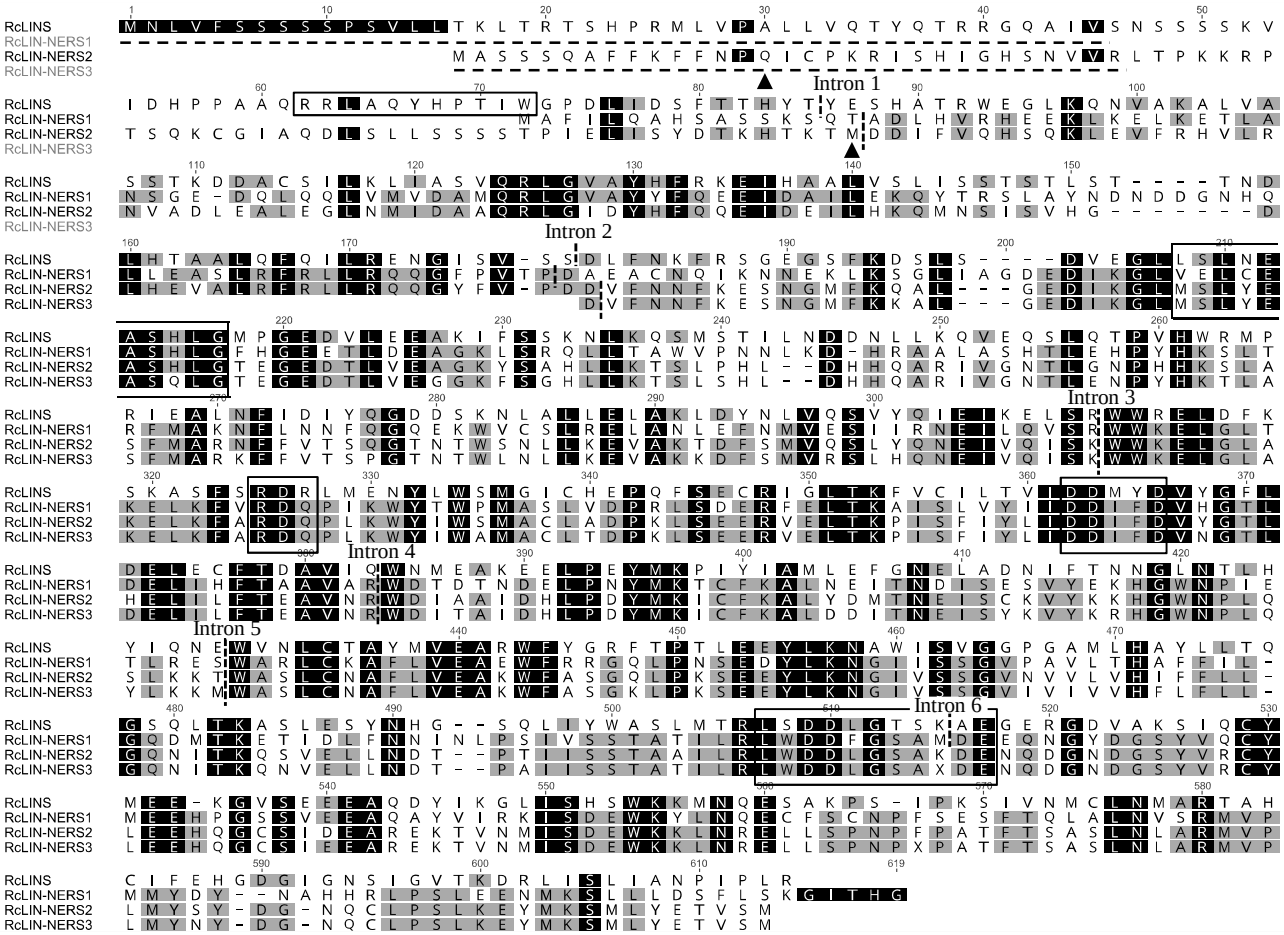
Figure 7: Representative results for GC-MS analysis of RcLINS in planta activity. Hexane extracted products synthesized by transformed tobacco plants were run on an apolar DB5 column. Ion m/z 93 was recorded in the SIM mode. A: profile for a control plant transformed by P19 alone. B: profile following cotransformation by RcLINS and P19. C: control trace (m/z 93) for a linalool standard. The experiment was repeated 5 (P19 control) or 7 (RcLINS plus P19) times with similar results.

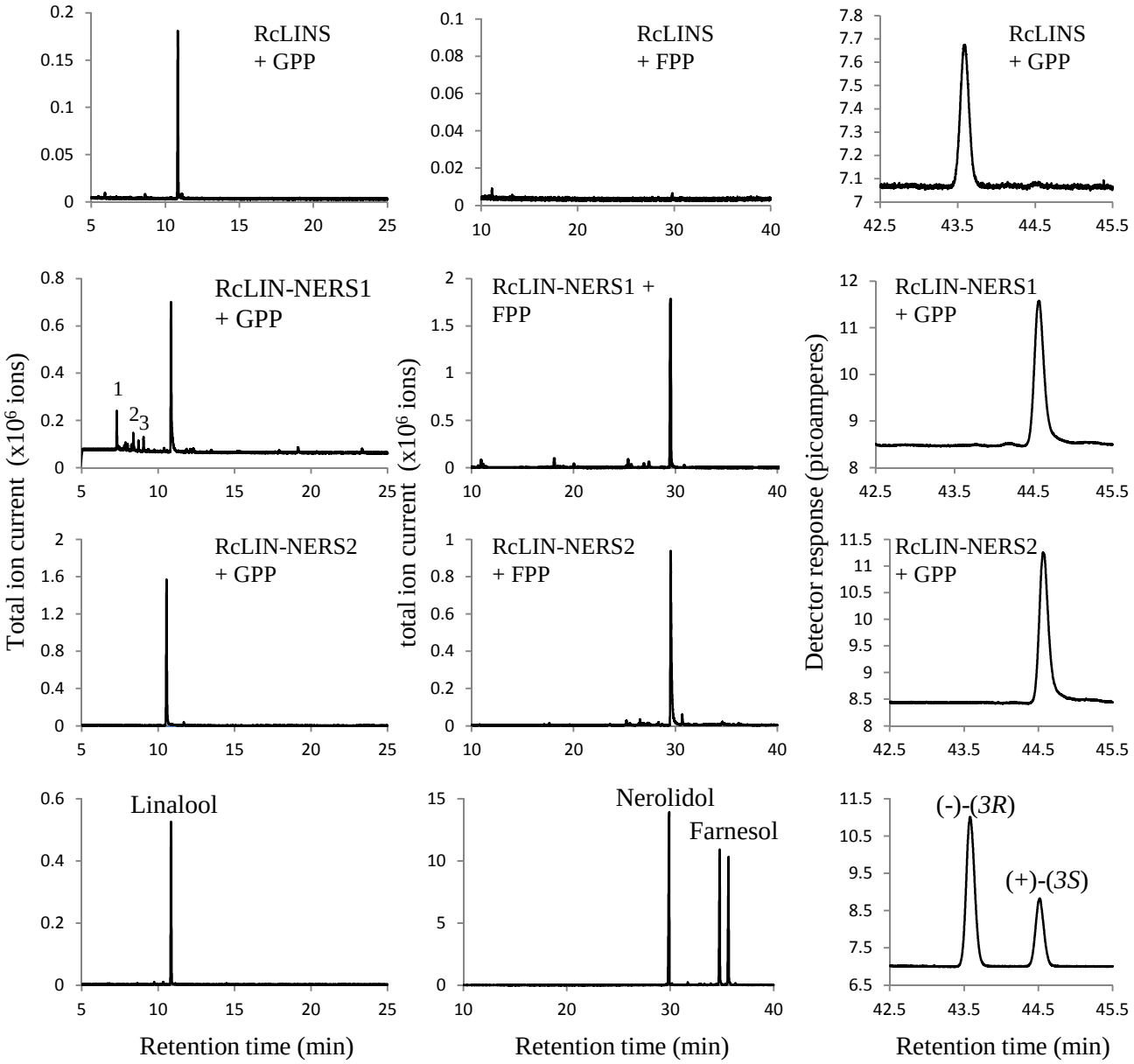
Figure 8: Confocal fluorescence microscopy of *Nicotiana benthamiana* cells expressing GFP fused proteins. A, B: leaves infiltrated with the RcLINS-GFP construct. B corresponds to the boxed section in A. C: infiltration with the SsLPPS *Salvia sclarea* diterpene synthase addressed to the stroma of the plastid (Caniard et al., 2012). D: control leaf infiltrated with the P19 viral suppressor of silencing alone. The GFP signal and the red signal corresponding to the autofluorescence of the chlorophyll are merged on all pictures.

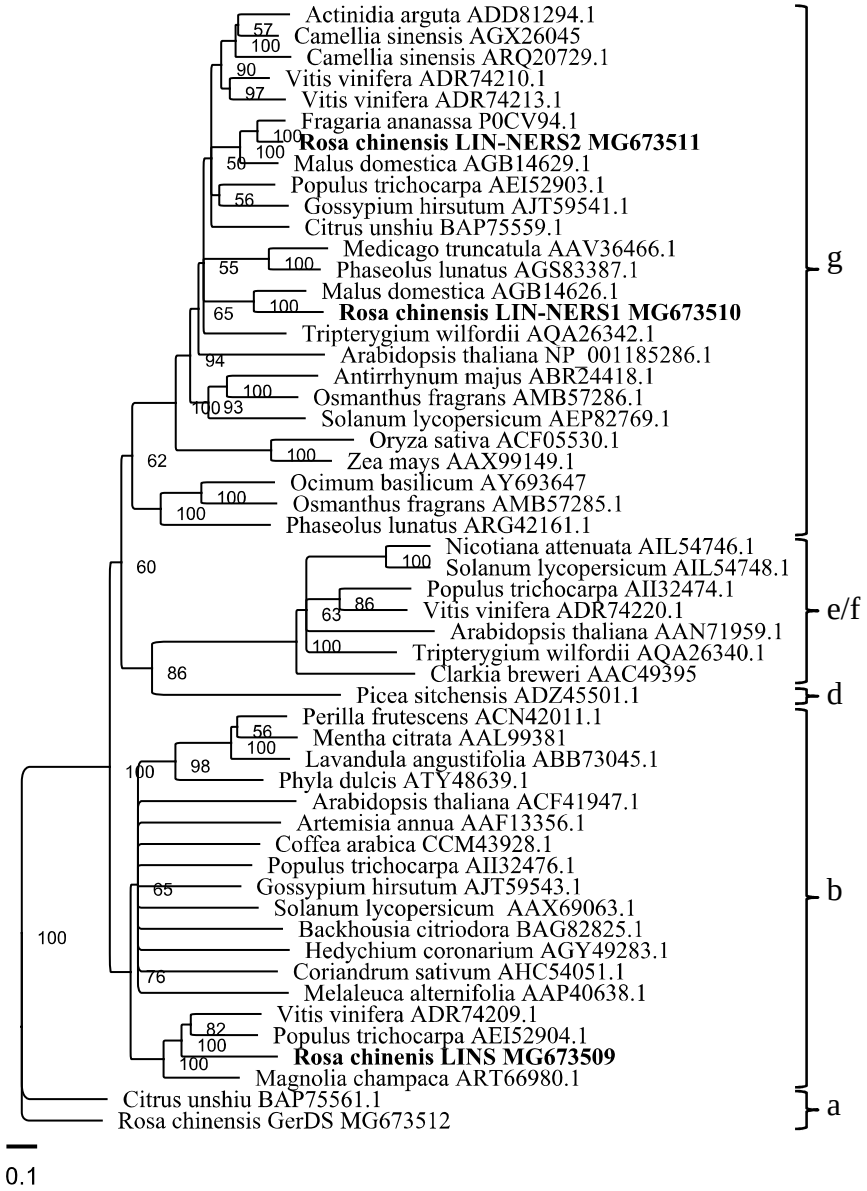
Supplementary Figure 1: Alignment of the RcLin-NerS2 sequences. The upper sequence in the alignment is the RcLIN-NERS2 sequence presented on Figure 1. The three other sequences correspond to highly similar genes that were cloned during the course of the genomic walk experiments. The arrowheads point respectively to the position of the internal Stop codon and to the alternative initiation methionine.

TPS	TPS subfamily	In vitro enzymatic activity	Localization
RcLINS MG673509	TPS-b	(-)-(*3R*)-linalool synthase	Plastidial
RcLIN-NERS1 MG673510	TPS-g	(+)-(*3S*)-linalool nerolidol synthase	Probable cytosolic
RcLIN-NERS2 MG673511	TPS-g	(+)-(*3S*)-linalool nerolidol synthase	Probable cytosolic
RcLIN-NERS3 MG673515	TPS-g	Probable pseudogene	

Table 1: Characteristics of rose linalool and linalool nerolidol synthases




RcLINS
+ GPP
RcLINS
+ FPP
RcLINS
+ GPP
RcLIN-NERS1
+ GPP
1
2
3
RcLIN-NERS1 +
FPP
RcLIN-NERS1
+ GPP
RcLIN-NERS2
+ GPP
RcLIN-NERS2
+ FPP
RcLIN-NERS2
+ GPP
Linalool
Nerolidol
Farnesol
(-)-(3R)
(+)-(3S)
Total ion current (x10⁶ ions)
total ion current (x10⁶ ions)
Detector response (picoamperes)
Retention time (min)
Retention time (min)
Retention time (min)


Actinidia arguta ADD81294.1
Camellia sinensis AGX26045
Camellia sinensis ARQ20729.1
Vitis vinifera ADR74210.1
Vitis vinifera ADR74213.1
Fragaria ananassa P0CV94.1
Rosa chinensis LIN-NERS2 MG673511
Malus domestica AGB14629.1
Populus trichocarpa AEI52903.1
Gossypium hirsutum AJT59541.1
Citrus unshiu BAP75559.1
Medicago truncatula AAV36466.1
Phaseolus lunatus AGS83387.1
Malus domestica AGB14626.1
Rosa chinensis LIN-NERS1 MG673510
Tripterygium wilfordii AQA26342.1
Arabidopsis thaliana NP_001185286.1
Antirrhynum majus ABR24418.1
Osmanthus fragrans AMB57286.1
Solanum lycopersicum AEP82769.1
Oryza sativa ACF05530.1
Zea mays AAX99149.1
Ocimum basilicum AY693647
Osmanthus fragrans AMB57285.1
Phaseolus lunatus ARG42161.1
Nicotiana attenuata AIL54746.1
Solanum lycopersicum AIL54748.1
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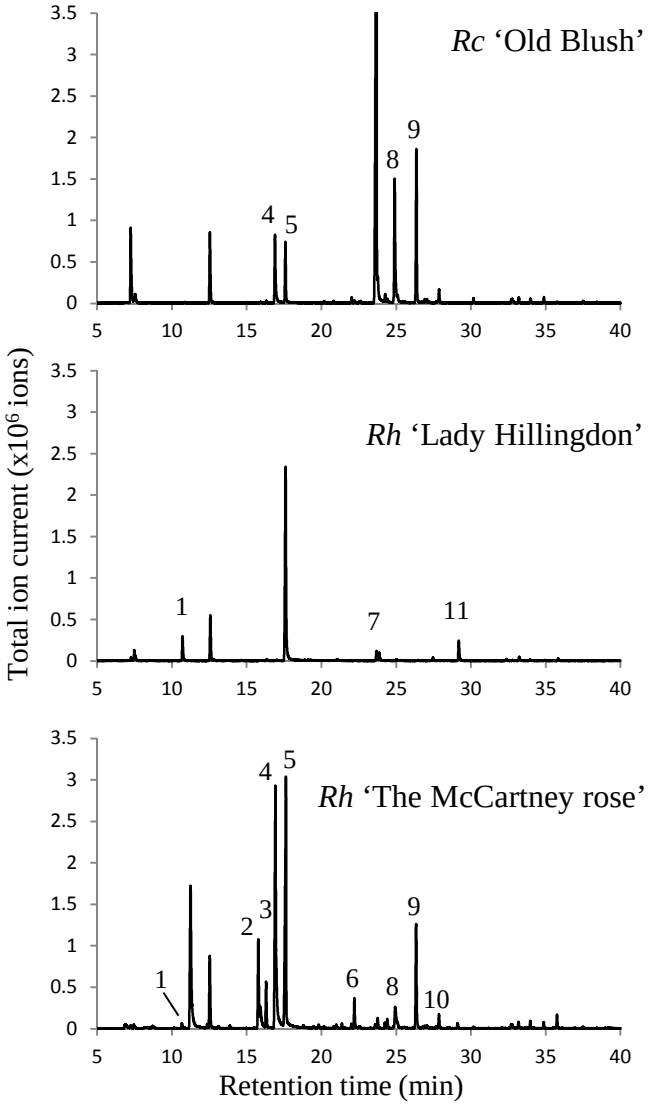
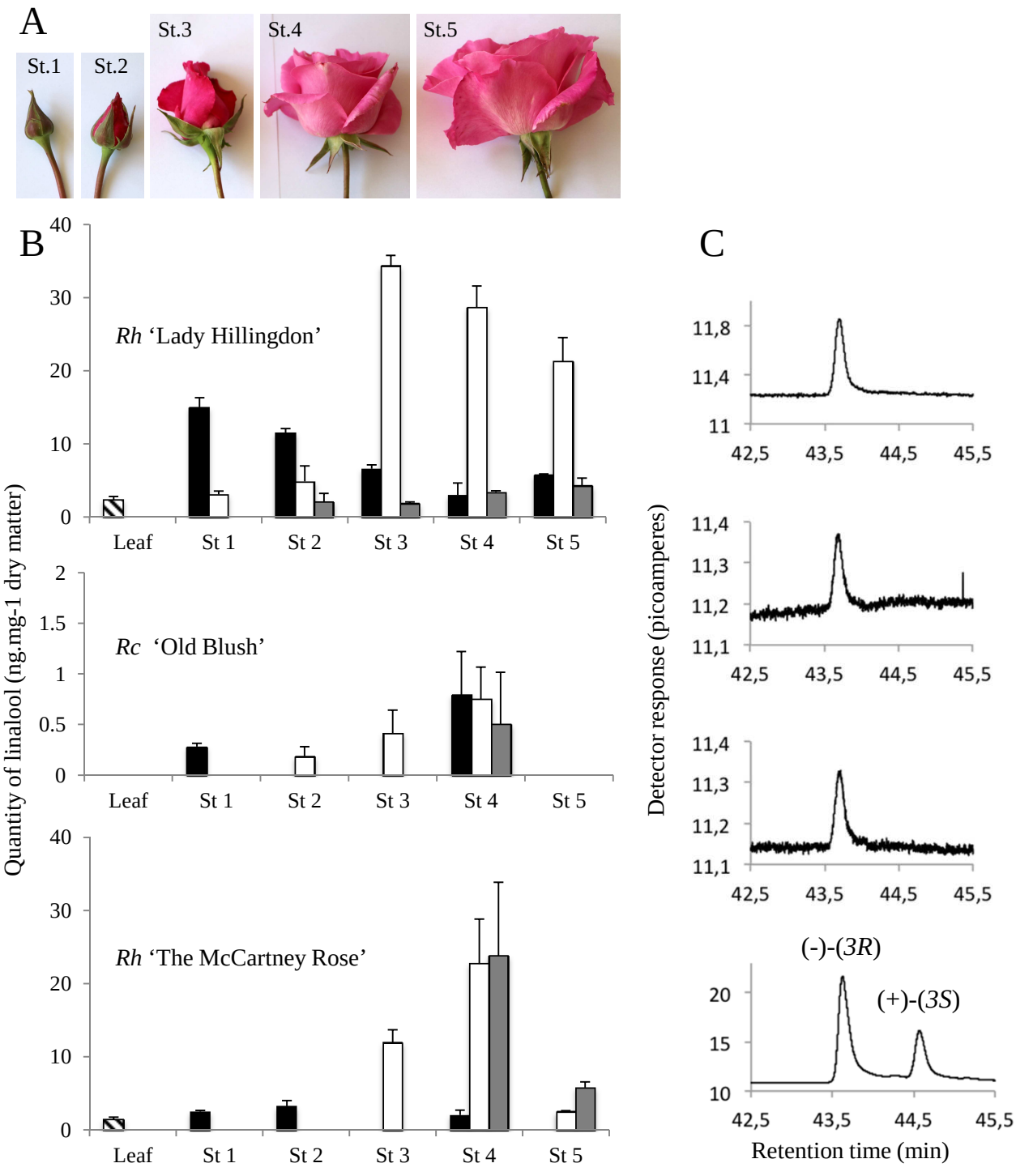
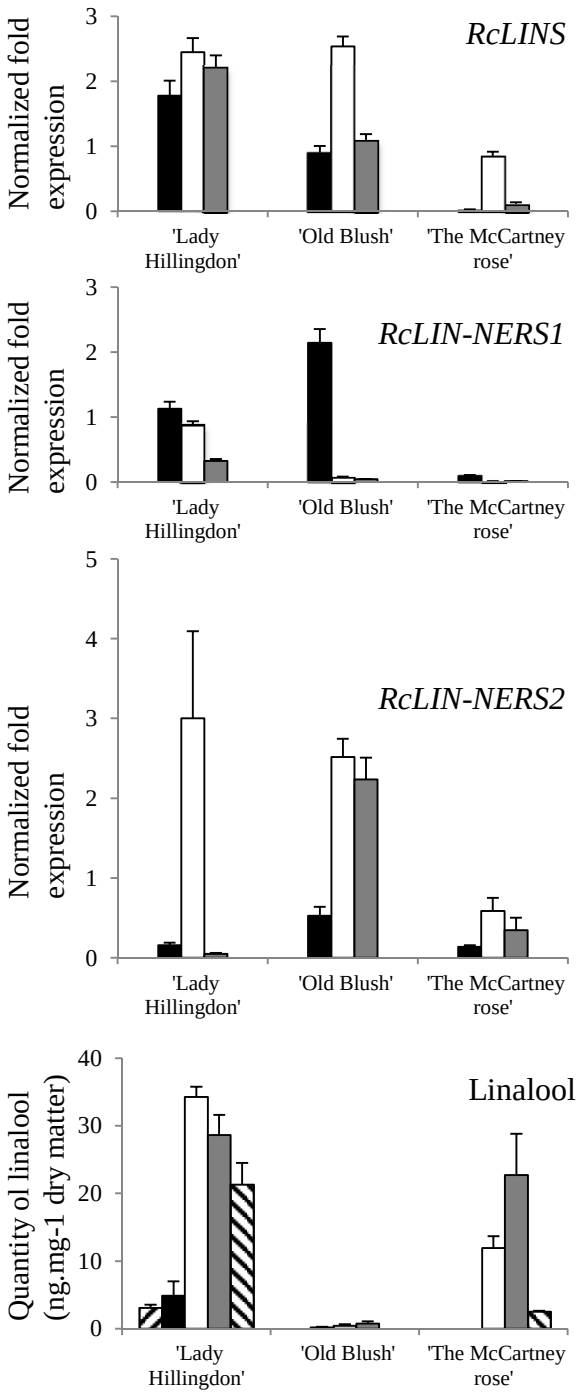
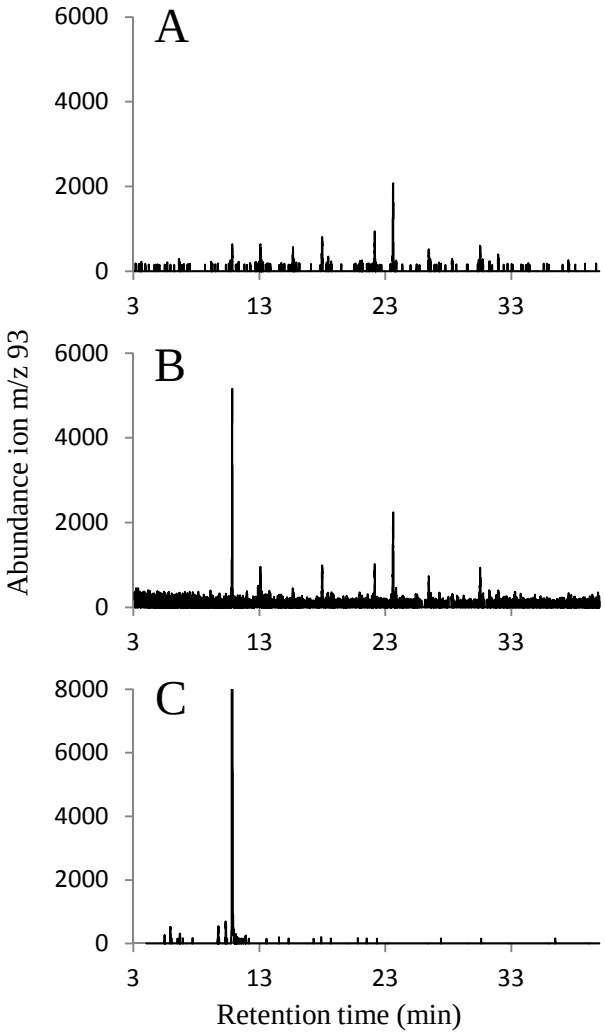
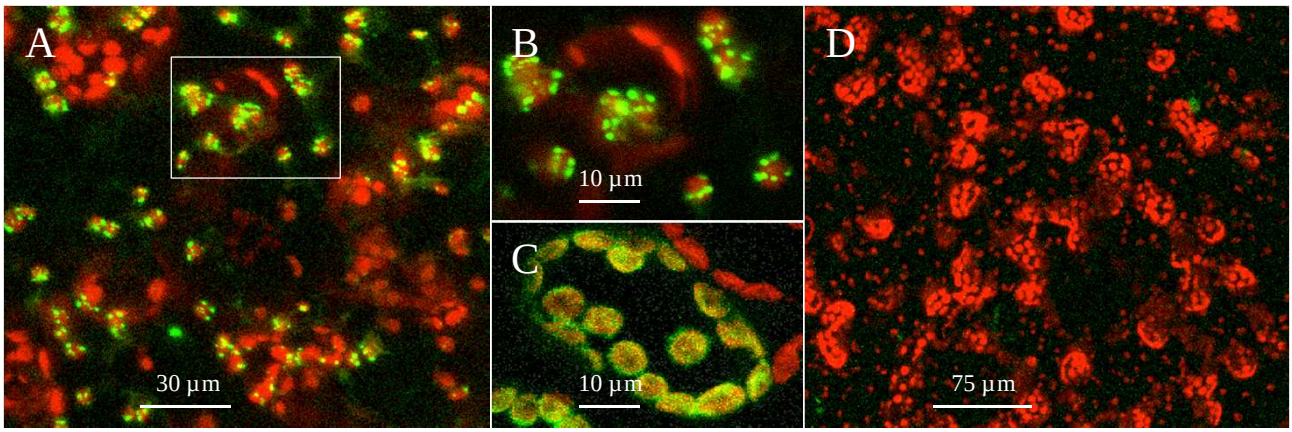
Vitis vinifera ADR74220.1
Arabidopsis thaliana AAN71959.1
Tripterygium wilfordii AQA26340.1
Clarkia breweri AAC49395
Picea sitchensis ADZ45501.1
Perilla frutescens ACN42011.1
Mentha citrata AAL99381
Lavandula angustifolia ABB73045.1
Phyla dulcis ATY48639.1
Arabidopsis thaliana ACF41947.1
Artemisia annua AAF13356.1
Coffea arabica CCM43928.1
Populus trichocarpa AII32476.1
Gossypium hirsutum AJT59543.1
Solanum lycopersicum AAX69063.1
Backhousia citriodora BAG82825.1
Hedychium coronarium AGY49283.1
Coriandrum sativum AHC54051.1
Melaleuca alternifolia AAP40638.1
Vitis vinifera ADR74209.1
Populus trichocarpa AEI52904.1
Rosa chinenis LINS MG673509
Magnolia champaca ART66980.1
Citrus unshiu BAP75561.1
Rosa chinensis GerDS MG673512
g
e/f
d
b
a
0.1


Rc ‘Old Blush’
Rh ‘Lady Hillingdon’
Rh ‘The McCartney rose’
Total ion current (x10^6 ions)
Retention time (min)


A
St.1
St.2
St.3
St.4
St.5
B
Quantity of linalool (ng.mg-1 dry matter)
Rh 'Lady Hillingdon'
Rc 'Old Blush'
Rh 'The McCartney Rose'
Leaf
St 1
St 2
St 3
St 4
St 5
C
Detector response (picoamperes)
(-)-(3R)
(+)-(3S)
Retention time (min)


RcLINS
Normalized fold expression
3
2
1
0
'Lady Hillingdon'
'Old Blush'
'The McCartney rose'
RcLIN-NERS1
Normalized fold expression
3
2
1
0
'Lady Hillingdon'
'Old Blush'
'The McCartney rose'
RcLIN-NERS2
Normalized fold expression
5
4
3
2
1
0
'Lady Hillingdon'
'Old Blush'
'The McCartney rose'
Linalool
Quantity ol linalool (ng.mg-1 dry matter)
40
30
20
10
0
'Lady Hillingdon'
'Old Blush'
'The McCartney rose'


A
B
C
6000
4000
2000
0
8000
3
13
23
33
Abundance ion m/z 93
Retention time (min)


A
30 µm
B
10 µm
C
10 µm
D
75 µm

Highlights:

- Four linalool and linalool nerolidol synthases have been characterized in roses.
- However, only one (-)-(*3R*)-linalool synthase participates to linalool production.
- Weak expression levels explain the low abundance of linalool in rose flowers.
- Rose monoterpenes are synthesized by both terpene synthases and a Nudix hydrolase.
- Respective implications of these pathways in monoterpene production is discussed.

Author contributions

Conception and design of the study: Jean-Louis Magnard, Frédéric Jullien, Sylvie Baudino. Realization of the experiments: Aurélie Rius Bony, Jean-Louis Magnard, Fabienne Bettini, Frédéric Jullien, Ausilia Campanaro, Bernard Blerot. Analysis of the data: Jean-Louis Magnard, Aurélie Rius Bony, Frédéric Jullien. Writing of the manuscript: Jean-Louis Magnard, Sylvie Baudino, Frédéric Jullien.