PLANT VOLATILES

Biosynthesis of monoterpene scent compounds in roses

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The scent of roses (*Rosa* x *hybrida*) is composed of hundreds of volatile molecules. Monoterpenes represent up to 70% percent of the scent content in some cultivars, such as the Papa Meilland rose. Monoterpene biosynthesis in plants relies on plastid-localized terpene synthases. Combining transcriptomic and genetic approaches, we show that the Nudix hydrolase RhNUDX1, localized in the cytoplasm, is part of a pathway for the biosynthesis of free monoterpene alcohols that contribute to fragrance in roses. The RhNUDX1 protein shows geranyl diphosphate diphosphohydrolase activity in vitro and supports geraniol biosynthesis in planta.

Roses are used as ornamental plants in gardens, as cut flowers, and as sources of essential oils for perfume and cosmetics. Breeding with a focus on cut flowers and visual attributes can leave scent traits disadvantaged (*1*). The cause for the lack of fragrance in these flowers is unknown and does not seem to be linked to increased vase life (*2*). Monoterpene alcohols and 2-phenylethanol characterize typical rose scents; volatile phenolic compounds characterize tea-scented roses (*3*). Although genes involved in the biosynthesis of phenolic scent compounds, 2-phenylethanol, and sesquiterpenes have been characterized (*4*–*6*), the basis for monoterpene biosynthesis remains obscure.

In plants, geranyl diphosphate (GPP), precursor of monoterpenes, is synthesized in plastids from dimethylallyl diphosphate and isopentenyl diphosphate supplied by the methylerythritol 4-phosphate pathway (*7*). The monoterpenes in essential oils are produced through the activity of various monoterpene synthases (*8*). For example, geraniol synthase (GES) converts GPP into geraniol in basil (*9*). Here we investigate monoterpene biosynthesis in roses. We used cDNA amplification fragment length polymorphism–differential display (AFLP-DD) and DNA microarrays to compare the transcriptomes of two rose cultivars that have different scents (see supplementary materials and methods).

The Papa Meilland (PM) cultivar emits a heavy typical rose scent, mostly composed of monoterpene alcohols and 2-phenylethanol. The Rouge Meilland (RM) cultivar produces very little scent and only trace amounts of these compounds (table S1). With AFLP-DD we identified two amplicons favored in PM, one with homology to the Nudix hydrolase family (DIF1) and one with homology to a laccase protein (DIF38) (table S2). With microarrays we found 91 genes expressed more in PM than in RM (data S1). The gene with the highest differential expression (PM1, 7583-fold increase in PM relative to RM) (table S2 and data S1) also corresponded to the Nudix hydrolase. We have named this gene *RhNUDX1* (GenBank accession number JQ820249). *RhNUDX1* encodes a 150–amino acid protein that contains the characteristic Nudix domain (*10*) and is similar (59% identity) to AtNUDX1 from *Arabidopsis thaliana* (fig. S1). In a survey of 10 rose cultivars with contrasting scent profiles (tables S1 and S2 and data S2), *RhNUDX1* expression correlated with the presence of monoterpene alcohols (geraniol, nerol, citronellol) and sesquiterpenes (farnesol, farnesene, and farnesyl acetate) (Fig. 1). Nudix hydrolases remove nucleoside diphosphates linked to other moieties (*10*). Some may also accept nonnucleoside substrates (*11*). These enzymes have various functions and may act as diphosphoinositol polyphosphate phosphohydrolases, coenzyme A pyrophosphatases, adenosine diphosphate–ribose pyrophosphatases, diadenosine polyphosphate hydrolases, and mRNA decapping enzymes (fig. S1B). Nudix hydrolyses are found in animals, plants, and bacteria. The number of Nudix representatives in each species varies from one in *Mycoplasma* to more than 50 in eukaryotes (*12*). In sequenced genomes of *Arabidopsis*, rice, and grapevine, the number of genes coding for putative NUDX proteins is 28, 33, and 30, respectively (*12*, *13*). RhNUDX1 shows the closest similarity to AtNUDX1 (fig. S1). This protein was proposed to have a similar function to *Escherichia coli* mutator protein (MutT), which acts to eliminate harmful compounds, such as 8-oxo–deoxyguanosine triphosphate (8-oxo-dGTP), which may be misincorporated in DNA during replication (*14*). We have searched rose transcriptome database (*15*) and identified 55 expressed sequence tags (ESTs) corresponding to putative NUDX genes, indicating that, like in other species, NUDX1 belongs to a gene family. All ESTs corresponding to *RhNUDX1* showed high expression levels in fully opened flowers (data S3). The other ESTs show no or weak expression levels in blooming flowers.

In PM, *RhNUDX1* was expressed in petals, where scent is produced, with little to no expression in stamens, sepals, or young leaves (Fig. 2). Expression increased at later stages of flower development (Fig. 2B, stages 3 to 5) when scent

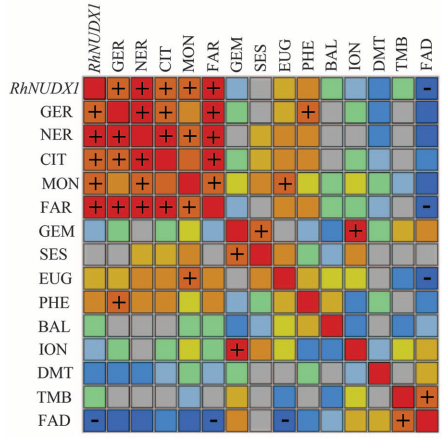

Fig. 1. Correlation map of the expression of *RhNUDX1* and quantity of scent compounds found in 10 rose cultivars. A nonparametric Spearman correlation test was used. Strengths of correlations are depicted by colors. Dark blue squares with minus signs indicate a significant negative correlation with correlation coefficient r close to −1 ($P < 0.05$). Dark red squares with plus signs indicate a significant positive correlation with r close to +1 ($P < 0.05$). BAL, benzyl alcohol and benzaldehyde; CIT, citronellol; DMT, 3,5-dimethoxytoluene; EUG, eugenol and methyleugenol; FAD, hexanal, *E*-2-hexenal, *Z*-3-hexenol, *E*-2-hexenol, 1-hexanol, *Z*-3-hexenyl acetate, hexyl acetate, and nonanal; FAR, *E*-α-farnesene, farnesol, farnesal, and farnesyl acetate; GEM, germacrene D, germacrene D-4-ol, and bicyclogermacrene; GER, geraniol, geranial, geranic acid, and geranyl acetate; ION, 3,4-dihydro-α-ionone and dihydro-α-ionol; MON, β-myrcene, *Z*-β-ocimene, and *E*-β-ocimene; NER, nerol and neral; PHE, 2-phenylethanol and phenylacetaldehyde; SES, δ-cadinene, elemol, α-cadinol, τ-cadinol, and τ-muurolol; TMB, 1,3,5-trimethoxybenzene.

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emission is at its maximum (*16*). RhNUDX1 protein accumulated at stage 4 in PM petals at the predicted molecular mass of 16.8 kD, but not in petals of the scentless cultivar RM (Fig. 2C). RhNUDX1 seems to carry no transit peptide, as evidenced by its sequence (*17*) and by cytosolic localization after transient expression in *Nicotiana benthamiana* of RhNUDX1 protein fused to green fluorescent protein (GFP) at its N or C terminus (fig. S2).

Expression of RhNUDX1 correlated with terpene biosynthesis. When we used an RNAi construct to knock down expression of *RhNUDX1* in *Rosa chinensis* cv. Old Blush (OB), a rose that produces high levels of the monoterpene geraniol, three independent transgenic events were generated, one of which (line A) showed reduced *RhNUDX1* expression (Fig. 3A). Volatile monoterpenes geraniol and geranial from petals were positively correlated with *RhNUDX1* expression levels (Fig. 3B and table S3). Line A had the least monoterpene content. Amounts of other volatiles were unaffected (fig. S3). To confirm the consequences of *RhNUDX1* down-regulation, we used a transient transformation strategy, which has been shown to allow efficient gene silencing in floral tissues (*18*). *Agrobacterium*-mediated transient transformation of rose petals was found to be extremely genotype-dependent. The best transformation efficiencies were obtained using the heavily scented genotype known as The Mac Cartney Rose, as shown by the expression of GFP (fig. S4). These petals were infiltrated with *Agrobacterium* harboring the *RhNUDX1* RNA interference (RNAi) construct used for stable transformation. Compared with petals expressing GFP, petals expressing the *RhNUDX1* RNAi construct had fewer monoterpenes; other classes of scent compounds were not significantly affected (fig. S5 and table S4).

To study the genetic basis of monoterpene biosynthesis in roses, we analyzed an F1 progeny from crosses between genotypes with different scent profiles. This mapping population consists of a full-sib family of 116 hybrids derived from a diploid cross between OB and a hybrid *Rosa wichurana* (RW) originating from the Bagatelle garden (Paris, France). The female parent (OB) produced high amounts of geraniol, whereas the male parent (RW) did not. *RhNUDX1* was expressed ~4000 times more in OB than in RW (fig. S6). Gas chromatography–mass spectrometry (GC-MS) analyses of petal volatiles showed that the geraniol content segregated in the progeny (fig. S7C). We detected a major quantitative trait locus (QTL) for geraniol biosynthesis on the female linkage group 2 (LG2). This QTL explained 76% of the observed variation in geraniol content (LOD score 39) (fig. S7B). When mapped on the female LG2, *RhNUDX1* colocalized with this QTL for geraniol production (fig. S7A). Together with genetic colocalization, the transgenic down-regulation data supports a role of RhNUDX1 in geraniol biosynthesis in roses.

Our hypothesis is that the RhNUDX1 protein, located in the cytosol, is in the biosynthetic pathway for monoterpenes. Recombinant RhNUDX1 failed to yield geraniol or farnesol after incubation with GPP and farnesyl diphosphate (FPP), suggesting that RhNUDX1 lacks terpene synthase activity (fig. S8). Recombinant RhNUDX1 (Fig. 4B) showed diphosphohydrolase activity when incubated with GPP and FPP. The products were geranyl monophosphate (GP) and farnesyl monophosphate (FP), respectively (Fig. 4A, fig. S9, and table S5). Optimal activity enzymatic activity occurred around pH 8, with very low activity below pH 6 (fig. S10). RhNUDX1 exhibited low Michaelis constant (K_m) values for GPP and FPP (140 and 480 nM, respectively) (table S5). The K_m for GPP was two orders of magnitude lower than that of GES (*9*) and in the same range as that of the MutT nudix protein of *E. coli* with its substrate 8-oxo-dGTP (*14*). Conversely, RhNUDX1 exhibited poor activity with 8-oxo-dGTP and dGTP.

To investigate RhNUDX1 involvement in geraniol biosynthesis, we compared its activity to that of GES from basil (*9*). We used *Agrobacterium*-mediated transient expression to express both in *N. benthamiana*. We verified that the RhNUDX1 protein was accumulated in tobacco leaves (fig. S11). Expression of GES in plastids resulted in the

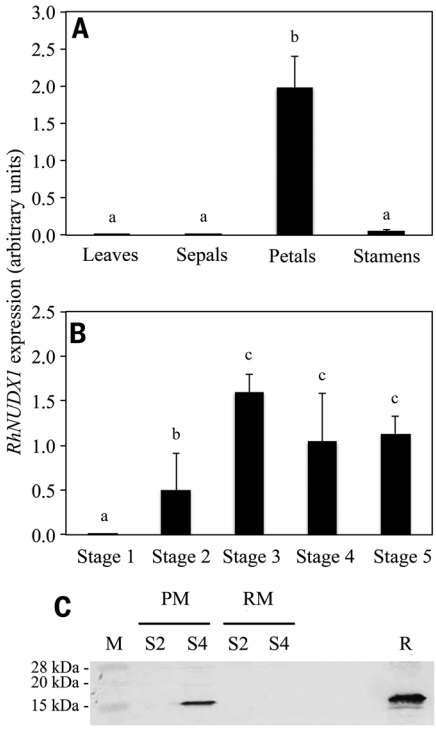

Fig. 2. Analysis of *RhNUDX1* expression in *Rosa* x *hybrida*. Flower developmental stages were defined as in (*16*). SE values are indicated by vertical bars (n = 6 replicates). Means with different letters (a, b, c) are significantly different (Tukey's HSD test, $P < 0.05$). (**A**) Expression in young leaves and floral organs at stage 4 (S4), analyzed by quantitative polymerase chain reaction (qPCR). (**B**) Expression in petals during development (from S1 to S5), analyzed by qPCR. (**C**) Western blot analysis of RhNUDX1 protein in *Rosa* x *hybrida* petals. M, Molecular weight marker; R, recombinant protein produced in *E. coli*.

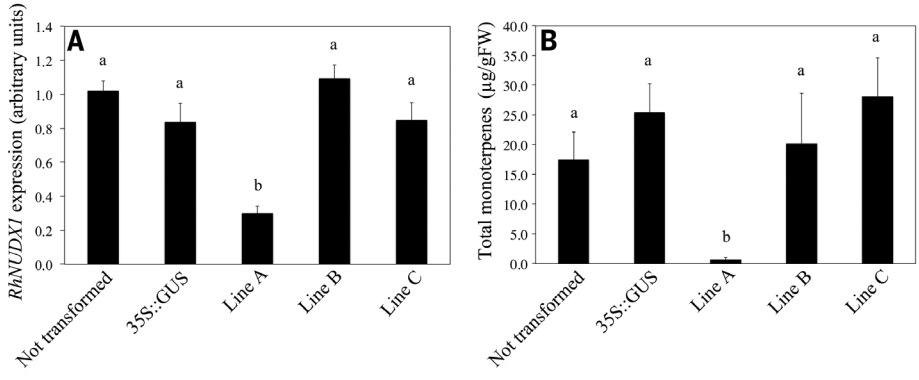

Fig. 3. Characterization of *RNAi-RhNUDX1* transgenic rose lines. (**A**) Real-time quantitative reverse transcription PCR analyses of *RhNUDX1* expression in petals of nontransformed plants, in plants transformed with the Gus reporter gene under the control of the 35*S* promoter (35*S*::GUS), and in three transgenic *RNAi-RhNUDX1* lines. SE values are indicated by vertical bars (n = 6 replicates). (**B**) GC-MS analyses of the petal volatile monoterpenes in *RNAi-RhNUDX1* transgenic rose lines. Amounts are expressed in micrograms per gram of fresh leaf weight (µg/g FW). SE values are indicated by vertical bars (n = 8 to 12 replicates). For both panels, means with different letters (a and b) are significantly different (Tukey's HSD test, $P < 0.05$).

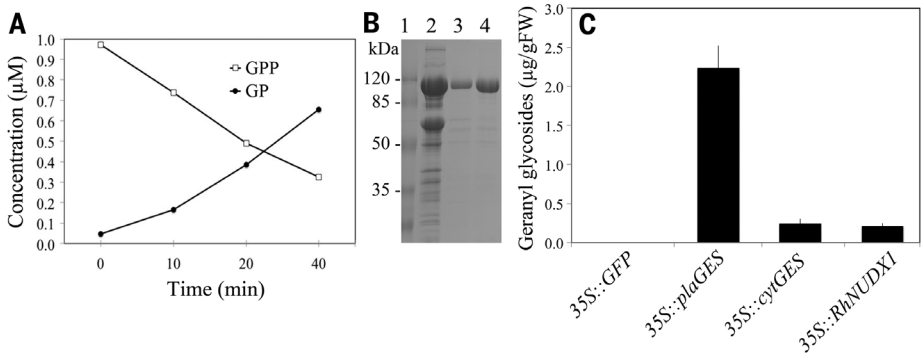

Fig. 4. Functional characterization of RhNUDX1 in vitro and in planta. (**A**) Decrease in GPP and concomitant increase in GP after incubation with recombinant NusA-RhNUDX1 fusion protein. NusA-RhNUDX1 (250 ng) was incubated in the presence of 1 μM GPP at 30°C. At the indicated time points, GPP and GP were quantified by liquid chromatography–mass spectrometry (LC-MS). (**B**) SDS–polyacrylamide gel electrophoresis analysis of recombinant NusA-RhNUDX1 protein. 1, molecular weight marker; 2, soluble protein extract from NusA-RhNUDX1–expressing *E. coli*; 3 and 4, purified NusA-RhNUDX1 protein. Molecular weights (in kilodaltons) are indicated. (**C**) Accumulation of geranyl glycosides after transient expression of *RhNUDX1* in *N. benthamiana*. GES from basil (*9*) was expressed as a full-length protein including its transit peptide (plastidic GES, 35*S*::plaGES) and as a truncated protein without transit peptide (cytosolic GES, 35*S*::cytGES). This latter construct was aimed at comparing GES and RhNUDX1 activity in a cytosolic context. GFP was used as a control. Geranyl glycosides were quantified by LC-MS 96 hours after transformation. Amounts are expressed in micrograms per gram of fresh leaf weight (μg/g FW) of geranyl glucoside equivalent, as means of triplicate assays. Bars indicate SE.

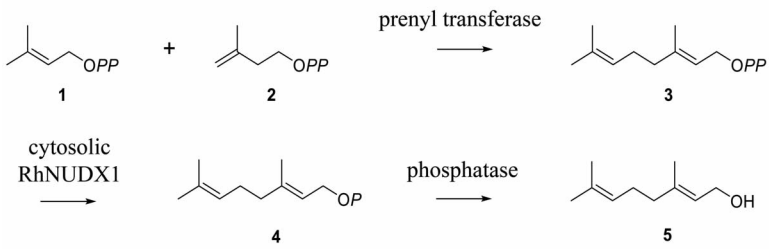

Fig. 5. Hypothetical biogenetic scheme for the formation of free geraniol in rose petals. 1, dimethylallyl diphosphate; **2**, isopentenyl diphosphate; **3**, GPP; **4**, GP; **5**, geraniol.

release of geraniol (fig. S12) and the accumulation of geranyl glycosides (Gglyc) (Fig. 4C). Expression of GES or RhNUDX1 in the cytosol both resulted in similar accumulations of Gglyc and geraniol (Fig. 4 and fig. S12). As RhNUDX1 did not dephosphorylate GP to geraniol in vitro (fig. S8), we assumed that enzymes (presumably phosphatases) carried out that conversion in planta. Indeed, we incubated rose petal proteins with GP and found that it was efficiently converted to geraniol (fig. S13). Although the RhNUDX1 enzyme was able to use FPP as a substrate in vitro, farnesol or farnesol glycoside did not accumulate in RhNUDX1-expressing tissues. Altogether, our data show that RhNUDX1 supports efficient geraniol and Gglyc biosynthesis in planta and that, in a cytosolic context, RhNUDX1 is equivalent to GES.

As GPP is a substrate for RhNUDX1, which is itself localized in the cytoplasm, we ask about the origin of GPP. GPP is a precursor for monoterpenes through pathways generally localized in plastids. Only a few studies suggest that GPP could be generated by the mevalonate pathway in some plants like rose and other Rosaceae (*19*–*22*). GPP for use in the cytosol for the biosynthesis of monoterpenes may be exported from plastids (*23*) or could also be a by-product of FPP synthase activity (*24*) or may be produced by a cytosolic GPP synthase.

We propose that RhNUDX1 is a cytosolic component of a terpene synthase–independent pathway for monoterpene biosynthesis that leads to scent production in roses (Fig. 5). The intensity of scent in roses may rely on RhNUDX1 and the monoterpenes it produces, and our finding may provide leverage to restore scent in roses with low levels of RhNUDX1 expression.

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SUPPLEMENTARY MATERIALS

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Material and Methods
Figs. S1 to S13
Tables S1 to S5
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Data S1 to S3

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Biosynthesis of monoterpene scent compounds in roses

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Stop to smell the roses

Some roses smell beautiful, yet others only look beautiful. Magnard *et al.* leveraged this distinction to study the biosynthesis of geraniol, a monoterpene alcohol in rose scent (see the Perspective by Tholl and Gershenzon). Enzymes known for geraniol synthesis in other plants, such as basil, did not seem to provide that function for roses. Instead, a diphosphohydrolase, which functions in the cytoplasm of cells in rose petals, generates the geraniol emitted by fragrant roses. Identification of the enzyme and its gene enables marker-assisted breeding to put the perfume back into beauty.
Science, this issue p. 81; see also p. 28