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Research article

Functional characterization of the eugenol synthase gene (*RcEGS1*) in rose

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ABSTRACT

The floral volatile compound eugenol is an important constituent in many aromatic plants, being a floral attractant for pollinators as well as having antimicrobial activity. Rose flowers emit eugenol and its derivatives. We recently reported a eugenol synthase gene (*RcEGS1*) (JQ522949) that was present in petals of *R. chinensis* cv. Old Blush. *RcEGS1* has its highest expression levels in the petals compared to other tissues; it has higher transcript levels at the developmental blooming stage and lower levels at budding and senescence stages. Here, we overexpressed the RcEGS1 protein in *Escherichia coli*, and showed by Western-blot analysis that its expression was mainly detected in stamens and petals at the flower opening stage. RcEGS1 was principally localized in the upper and lower epidermal layers, which are the major sites of scent emission in roses. Furthermore, we demonstrated that down-regulation of *RcEGS1* expression in flowers by virus-induced gene silencing led to a reduction of the relative content of eugenol. We suggested that *RcEGS1* was responsible for eugenol biosynthesis in roses.

1. Introduction

Phenylpropenes such as eugenol, chavicol, estragole and anethole make up the unique properties of many herbs and spices (Grossman, 1993). In flowers, they attract pollinators and are used as defense compounds against fungi and bacteria (Prasad et al., 2004; Atkinson, 2017). Many plants synthesize volatile phenylpropenes in their floral and vegetable organs as a defense against insect pests and herbivores (Grossman, 1993; Obeng-Ofori and Reichmuth, 1997; Koeduka et al., 2008). The flowers of *Clarkia breweri* emit a mixture of volatiles that contain eugenol, isoeugenol, methyleugenol, and methylisoeugenol (Raguso and Pichersky, 1995). Basil (*Ocimum basilicum*) varieties synthesize and accumulate high levels of eugenol in the glands at the surface of their leaves and flowers (Koeduka et al., 2008; Gang et al., 2001). Flowers of *Petunia hybrida* produce high levels of isoeugenol and smaller amounts of eugenol, which are isomeric forms *in planta* (Verdonk et al., 2003). Eugenol is also found in significant amounts in spice plants such as clove buds, cinnamon and pepper (Hong et al., 2015; Grush et al., 2004; Guenther, 1949). Moreover, phenylpropenes have been detected in a range of important fruits (Atkinson, 2017). Some rose cultivars, for example *Rosa* x *damascena* and *Rosa* x *centifolia*, also produce eugenol and its derivatives, especially in their stamens (Rusanov et al., 2011).

Genes involved in the biosynthesis of phenylpropenes have been characterized in many plants (Koeduka et al., 2014). Petunia IGS1 (PhIGS1) and Basil EGS1 (ObEGS1) catalyze the formation of eugenol and IGS1 catalyzes the formation of isoeugenol using coniferyl acetate as a substrate (Koeduka et al., 2006). *C. breweri* flowers have two eugenol synthases (*CbEGS1*, *CbEGS2*) and one isoeugenol synthase (*CbIGS1*) genes that are closely related to each other. The three genes use the same substrate coniferyl acetate to make a single product, eugenol or isoeugenol, although their catalytic efficiencies may differ to some extent (Koeduka et al., 2008). In addition, *PhIGS1* is expressed specifically in floral tissues, mostly in the upper and lower parts of the petals (corolla and tube). These tissues are responsible for most of the scent produced. In basil, the ObEGS1 protein appears to be restricted to glands, where eugenol or isoeugenol are synthesized and stored (Koeduka et al., 2008).

In a previous study, a eugenol synthase gene (*RcEGS1*) was cloned from the petals of *R. chinensis* cv. Old Blush by using PCR (Wang et al., 2012). Here, *RcEGS1* was overexpressed in *E. coli* and the protein was purified, which allowed the study of its expression in different organs by Western-blot analysis. Furthermore, VIGS technology was used to silence *RcEGS1* in rose petals and the amounts of scent compounds were analyzed by GC/MS in these *RcEGS1*-silenced plants. The results suggest that *RcEGS1* is involved in the biosynthesis of eugenol in roses.

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2. Materials and methods

2.1. Plant material

R. chinensis cv. Old Blush and *R.* x *hybrida* cv. Yunxiang were grown at the rose germplasm garden of the Flower Research Institute, Yunnan Agriculture Academic Science, Kunming, China. Flowers at five different developmental stages, described by Guterman et al. (2002), were picked and frozen immediately in liquid nitrogen then stored at −80 °C until use.

2.2. Heterologous expression of recombinant protein RcEGS1 and western-blot analysis

The plasmid containing *RcEGS1* was double-digested with *SmaI* and *SalI*, and inserted into pET28b pre-digested with the same enzymes to obtain pET28b-*RcEGS1*. The combined plasmid was then transformed into *E. coli* BL21 cells (TakaRa, China) in LB media containing 100 mg/L kanamycin and positive colonies were verified using DNA sequencing. The expression of recombinant proteins was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 3 h, until an OD at 600 nm of 0.4–0.6 was reached. Bacterial cultures were collected by centrifugation at 5000 × g and 4 °C for 10 min and re-suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl with 10 mM imidazole, pH 8.0) before being treated by sonication. The lysate obtained was centrifuged at 12,000 × g for 10 min at 4 °C. Induced proteins were purified using Ni-NTA affinity chromatography (Sangon Biotech, Shanghai, China). The samples (10 μg) and the protein marker (5 μg) were loaded onto a 12% SDS-PAGE gel to validate both protein expression and purification.

For western blots, 'Old Blush' RcEGS1 produced in *E. coli* and purified was extracted from gels and used to obtain polyclonal antibodies (Sangon Biotech, Shanghai China). Anti-EGS1 antibodies were used at a 1: 3000 dilution and incubated with the protein gel blots for 1 h. For the other conditions of the western blots, the protocol of (Fridman et al., 2005) was followed.

2.3. Immunolocalization

Petals of *R.* x *hybrida* cv. Yunxiang were quickly fixed overnight at 4 °C in 3.7% formaldehyde, 5% acetic, 50% ethanol. Tissue preparation with dimethylbenzene, dehydration, clearing, and wax embedding were performed according to the protocol of Sangon Biotech (Shanghai China). Antigen retrieval and addition of primary and second antibodies (anti-rabbit) were also done according to the protocol of Sangon Biotech (Shanghai China). Other conditions of immunolocalization were carried out as reported previously in rose (Scalliet et al., 2006).

2.4. Vector construction and vacuum infiltration

For the silencing of *RcEGS1*, a 167 bp fragment in the 3′ end of *RcEGS1* was obtained by gene synthesis (Sangon Biotech, Shanghai China), and subsequently cloned into the pTRV2 vector to generate the expression vector pTRV2-*RcEGS1*. The sequence of the 3' end of *RcEGS1* is listed in Supplementary file 1.

To establish virus-induced gene silencing (VIGS) in rose, vacuum infiltrations were performed as described previously (Tian et al., 2014). To this end, the *Agrobacterium* strain DHA105 containing TRV vectors (Ma et al., 2008) was grown at 28 °C in YEB medium containing 10 mM MES and 20 mM acetosyringone with appropriate antibiotics. After 24 h, *Agrobacterium* cells were harvested and re-suspended in the *Agrobacterium* infiltration buffer (10 mM MgCl2, 10 mM MES, pH 5.6, 150 mM acetosyringone). The sprouts were cut from the plants, submerged in an infiltration mixture containing pTRV1 and pTRV2 (OD_{600} = 1.0) and subjected to a vacuum at −25 kPa twice, for 60 s each. The treated plants were washed with distilled water and then grafted onto appropriate rootstocks of *R.* x *hybrida* cv. Yunxiang. The grafting method was performed according to the protocol of (Yan et al., 2018).

2.5. Identification of the volatile compounds of the rose flowers

Volatiles were collected for 45min from opening flowers by solid-phase micro-extraction (SPME) methods, using a 75 μm CAR/PDMS SPME fiber (Supelco, Bellefonte, PA, USA). After extraction, the compounds of rose petals were analyzed according to Yan et al. (2011). Three biological replicates of three grafted roses were used for each analysis.

3. Results

3.1. Production of recombinant RcEGS1 enzyme in E. coli

We previously cloned a putative eugenol synthase gene (*RcEGS1*, JQ522949) from the petals of *R. chinensis* cv. Old Blush (Wang et al., 2012). In this study, the coding regions of *RcEGS1* were inserted into the *E. coli* expression vectors pET28b, and transformed into the *E. coli* strain BL21 to obtain recombinant proteins. Recombinant protein pET28b-*RcEGS1*was produced by induction with 0.5 mM IPTG at 16, 20, 25, 30, 37 °C. The proteins in cells and in the supernatant were collected and examined on a 12% SDS-PAGE electrophoresis (Fig. 1A). The protein expression conditions were optimized to improve production. SDS-PAGE analysis showed that the protein RcEGS1 was successfully expressed in the total cell extract at 37 °C, with a molecular weight of approximately 35 kDa, while the *E. coli* DE3/RcEGS1 proteins were not found at the expected position (around 35 kDa) in the uninduced sample (Fig. 1A).

After the cultivation period, cells were harvested via centrifugation. The supernatants containing recombinant RcEGS1 proteins were applied to the Ni-NTA affinity column and subsequently purified using ion exchange chromatography (Sangon Biotech, Shanghai, China). The target fractions were then combined and incubated with PBS (pH7.2 ~ 7.4) to cleave the His-tag from RcEGS1. Finally, the recombinant protein was subjected to size-exclusion chromatography. The final purified RcEGS1 preparation yielded only one major protein band, at 35 kDa, after analysis by SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 1B).

3.2. RcEGS1 expression is regulated during flower development

To investigate RcEGS1 protein expression and know whether it correlates with the emission of floral volatiles, the purified recombinant RcEGS1was used to immunize rabbits. We used the anti-RcEGS1 antibody to determine the abundance of RcEGS1 proteins in different tissues from 'Old Blush', including petals from flowers at different developmental stages (stages 3, 4, and 5, according to Guterman et al., 2002), sepals and stamens from flowers at stage 3 and leaves. After western blot analyses, only one major band of the expected size was obtained, suggesting that the protein is present in one major isoform in the plant. However, it cannot be completely ruled out that other protein isoforms, not recognized by the antibody, or that cannot be separated by SDS-PAGE, are present in the rose petals. Western-blot analysis showed that RcEGS1 was found only in floral tissues, mostly in the stamens and the petals of 'Old Blush' (Fig. 2A). These tissues are responsible for most of the production of floral volatiles, including eugenol (Verdonk et al., 2003; Bergougnoux et al., 2007). We then analyzed the expression pattern of RcEGS1 protein at five flower developmental stages. RcEGS1 protein was not detected at stages 1 and 5 in 'Old Blush' flowers. A low level of RcEGS1 was detected at stage 2, and RcEGS1 protein accumulation attained its maximum at stages 3 and 4 (Fig. 2B), when scent emission was at its peak. It was followed by a decrease of protein to the background level at stage 5, when volatile emission was at its lowest level.