Short Communication

Expression of genes encoding protein kinases during flower opening in two cut rose cultivars with different longevity

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Abstract
Ethylene plays an important role in wide-ranging aspects of plant growth and development, including fruit ripening, leaf and flower senescence. In this study, the expression patterns of two genes involved in the ethylene signal transduction pathway (*RhCTR1* and *RhCTR2*) were investigated during the flower opening stages in two *Rosa hybrida* cultivars, 'Black magic' and 'Maroussia', which are characterized by short and long vase lives, respectively. *RhCTR1* expression increased significantly during flower opening in both cultivars, but its expression level in cv. Maroussia was significantly higher than that in cv. Black magic. No variation in gene expression was detected for *RhCTR2* in both cultivars. Therefore, this study showed that the vase life of the two cultivars correlated with the expression of *RhCTR1*, but not with that of *RhCTR2*, the behavior of which is typical of a constitutive gene.
Keywords: Cut rose (*Rosa hybrida*); Ethylene sensitivity; Gene expression; *RhCTR1* and *RhCTR2*

Rose is one of the most important cut-flowers in ornamental plants. For the purpose of commercial production, cut roses are usually harvested at a specific bud stage. Depending on the cultivar and the handling conditions, the flowers often do not open (Tan *et al*., 2006). The gaseous hormone ethylene plays an important role in diverse aspects of plant growth and development, ranging from seed germination (Abeles *et al*., 1992) to senescence and abscission of flowers, fruits and leaves (Giovannoni, 2001; Johnson and Ecker 1998). Also, in ornamental plants, ethylene influences flower opening (Reid *et al*., 1989), petal senescence (Nukui *et al*., 2004; Shibuya *et al*., 2000) and abscission (Kuroda *et al*., 2003). Current evidence indicates that ethylene is sensed by a small family of receptors, the Ethylene Responsive Elements (ETRs), which are able to form a complex with a kinase, namely *CTR1*. In the absence of ethylene, this complex actively represses the ethylene response pathways. The receptor/*CTR1* complex appears to operate by negatively regulating *EIN2* which is thought to activate response pathways through the *EIN3* family of transcriptional regulators (Bleeker, 2001). The loss-of-function *CTR1* mutants result in a constitutive ethylene-response phenotype, indicating that *CTR1* negatively regulates the ethylene response pathway (Chang and Stadler, 2001; Kieber *et al*., 1993). Therefore, *CTR1* is a key gene in the ethylene signal transduction pathway. In tomato, unlike *Arabidopsis thaliana*, which only contains one constitutively expressed *AtCTR1* gene, a family of four

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CTR1-like genes (*LeCTR1-LeCTR4*) has been identified (Lin *et al*., 1998), and expression analysis has revealed that the expression of *LeCTR1* increases during fruit ripening and flower senescence (Leclercq *et al*., 2002; Zegzouti *et al*., 1999), whereas *LeCTR2* is expressed constitutively (Alexander and Grierson, 2002). A homologue of *CTR1* has been cloned and characterized in *Cucurbita pepo,* and during flower development, *Cup-CTR1* is up-regulated in male flowers but not in female flowers (Manzano *et al*., 2008).

The induction of petal senescence or abscission by ethylene or pollination is associated with transcriptional regulation of the *ACS* and *ACO* (Fernàndez-Otero *et al*., 2006; Jones, 2003) and ethylene receptor genes (such as *DC-ERS1*, *DC-ERS2* and *DC-ETR1* in *Dianthus caryophyllus* L. and *Dl-ERSI* type-1 and *Dl-ERSI* type-2 in *Delphinium*) (Kuroda *et al*., 2003; Shibuya *et al*., 2002). Besides, this induction is also accompanied with an increase in the *CTR* genes of some ornamental plant species (Kuroda *et al*., 2004; Müller *et al*., 2002). Flower opening and senescence in roses is sensitive to ethylene, although the degree of this sensitivity varies in different cultivars (Müller *et al*., 2001; Reid *et al*., 1989). In miniature potted roses, this difference could be due to the different expression levels of receptor genes rather than the ethylene biosynthetic genes (Müller *et al*., 2000 a,b). The aim of this study was to evaluate the expression pattern of two critical genes (*RhCTR1* and *RhCTR2*) in the ethylene signal transduction pathway for a better understanding of the principles of gene regulation during rose flower senescence.

Rosa hybrida cv. Maroussia (long vase life) and cv. Black magic (short vase life) were grown in a hydroponic greenhouse. Petal samples were collected at 2 stages of rose flower opening (stage 2: partially opened bud; stage 8: fully-opened flower), as described by Wang *et al*. (2004). Petal samples were immediately detached and frozen in liquid nitrogen and stored at -80ºC for eventual total RNA isolation. Rose petals from each stage (Fig. 1) were grounded by means of the high-speed mixer mill (MM301, Retsch, Haan, Germany). High-quality total RNA was successfully isolated from 100 µg of petal tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Total RNA was quantified by spectrophotometric analysis using a Biophotometer (Eppendorf, Germany).

First-strand cDNA was synthesized from 5 µg of total RNA using the ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, UK) following the manufacturer's instructions. cDNA was quantified using the Biophotometer.

Real-time PCR was carried out by the Applied Biosystems 7500 Fast Real-Time PCR system using the DYNAmo HS SYBR Green qPCR kit (Finnzymes, Finland), in accordance with the recommendations of the manufacturer. Reaction mixtures (30 µl) contained 15 µl of 2 × SYBR-Green mixed with 0.5 µl of Rox solution, 1 µl each of 10 µM forward and 10 µM reverse primers, 5 µl of cDNA and 7.5 µl of distilled water. Primers' sequences, as reported by Müller *et al*. (2000 a,b), are presented in Table 1. The thermal profile used consisted of 2 min at 50ºC, 10 min at 95°C and 40 repeats of denaturation at 95°C for 15 s and annealing-extension-fluorescence data acquisition at 60°C for 1 min. In a 96-well plate, each reaction was performed in triplicate. An endogenous 18S rRNA was used as an internal standard. Relative expression levels were calculated using the delta (Δ) threshold cycle (Ct) method (Applied Biosystems). Data from quantitative PCR were subjected to ANOVA using Statistica8.0 package (StatSoft Inc., Tulsa, OK, USA). A randomized complete block design with three independent biological replications was used. 40- delta ΔCt was taken as an dependent variable. Means were separated using the least significant difference test (LSD) at the 0.01 level of probability.

A significant increase ($P<0.01$) was observed for the expression level of *RhCTR1* between the two flower opening stages, in both rose cultivars (Fig. 1 and Table 2). Analogous results were observed in tomato during fruit ripening and flower senescence (Leclercq *et al*., 2002; Zegzouti *et al*., 1999), and in the miniature potted roses (Müller *et al*., 2002) and two cut rose cultivars (Tan *et al*., 2006) during flower

Table 1. Primer sequences of *CTR1*, *CTR2* and 18S rRNA.

Gene	Forward primer	Reverse primer
CTR1	5'- GAT GGC GCC AGA AGT CC-3'	5'- GCC CAG CAA GCC TCA AT-3'
CTR2	5'- GTC GCG CTT GAA ACA TAA CA -3'	5'- AAC AGG GGG ATC AAC TTC TTT-3'
18S rRNA	5'- CGG GGA GGT AGT GAC AAT AAA TAA CA-3'	5'- CCA CCA CCC ATA GAA TCA AGA AAG AG-3'

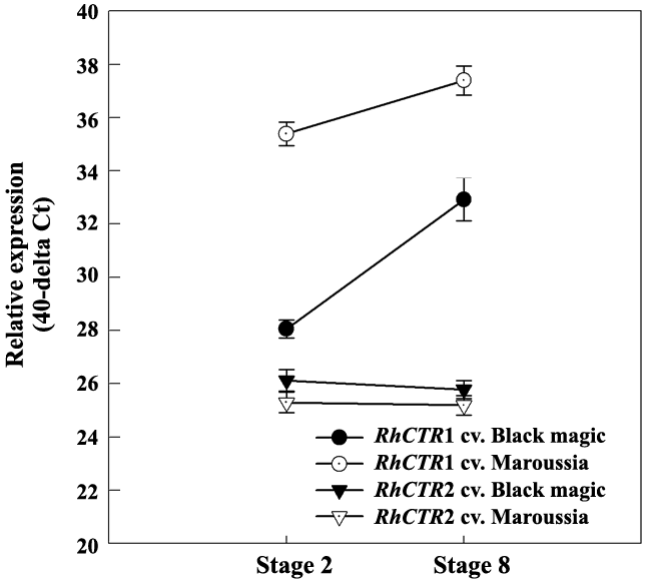

Figure 1. Expression level of *RhCTR* genes in two cut roses cv. Black magic and cv. Maroussia between two stages of flower opening. The vertical bar is the least significant difference (LSD; P<0.01), for measuring significant difference between cultivars.

Table 2. Regression slope and percentage variation of *RhCTR* gene expression between two stages of flower opening in two cut roses cv. Black magic and cv. Maroussia.

Gene	Cultivar	Slope	Percentage	*P*-value
RhCTR1	Black magic	4.8	17.3%	P<0.01
	Maroussia	2.0	5.7%	P<0.05
RhCTR2	Black magic	-0.4	-1.3%	ns
	Maroussia	-0.2	-0.4%	ns

senescence. The expression level of *RhCTR1* was higher in cv. Maroussia when compared to that in cv. Black magic, but the increase in expression of this gene, through stages 2 and 8, was greater in cv. Black magic in comparison to cv. Maroussia (Fig. 1 and Table 2). This suggests that the vase life might be reduced by increasing the level of expression of *RhCTR1* during the different flower opening stages inspite of its expression level. Further studies will be needed to confirm this hypothesis.

Results also suggest that cv. Maroussia may not be sensitive to ethylene according to previous experiments (unpublished data), since the standard model for the ethylene signal transduction pathway confirms that a high level of *CTR* expression should result in decreased ethylene sensitivity (Tan *et al*., 2006). These findings are consistent with other studies in *Arabidopsis thaliana* (Hua and Meyerowitz, 1998 a), tomato (Tieman *et al*., 2000), and carnation (Shibuya *et al*., 2002), supporting the role of ethylene receptors in the negative regulation model (Chen *et al*., 2005; Hua *et al*., 1998 b).

The comparison of the expression level of *RhCTR2* between the two cultivars revealed no significant difference, as shown in Figure 1 and Table 2. No significant difference was also found for *RhCTR2* expression between the two stages of the flower opening stages. Furthermore, results showed that expression levels of *RhCTR2* were lower than that of *RhCTR1*. Therefore, it can be hypothesized that the behavior of this gene is similar to that of a constitutive gene as previously observed in other studies on two cut rose cultivars (Tan *et al*., 2006) and miniature potted roses (Müller *et al*., 2002).

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