Comparison of mRNA levels of three ethylene receptors in senescing flowers of carnation (Dianthus caryophyllus L.)

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Abstract

Three ethylene receptor genes, DC-ERS1, DC-ERS2 and DC-ETR1, were previously identified in carnation (Dianthus caryophyllus L.). Here, the presence of mRNAs for respective genes in flower tissues and their changes during flower senescence are investigated by Northern blot analysis. DC-ERS2 and DC-ETR1 mRNAs were present in considerable amounts in petals, ovaries and styles of the flower at the full-opening stage. In the petals the level of DC-ERS2 mRNA showed a decreasing trend toward the late stage of flower senescence, whereas it increased slightly in ovaries and was unchanged in styles of the flower at the full-opening stage. In the petals the level of DC-ERS2 mRNA showed a decreasing trend toward the late stage of flower senescence, whereas it increased slightly in ovaries and was unchanged in styles throughout the senescence period. However, DC-ETR1 mRNA showed no or little changes in any of the tissues during senescence. Exogenously applied ethylene did not affect the levels of DC-ERS2 and DC-ETR1 mRNAs in petals. Ethylene production in the flowers was blocked by treatment with 1,1-dimethyl-4-(phenylsulphonyl)semicarbazide (DPSS), but the mRNA levels for DC-ERS2 and DC-ETR1 decreased in the petals. DC-ERS1 mRNA was not detected in any cases. These results indicate that DC-ERS2 and DC-ETR1 are ethylene receptor genes responsible for ethylene perception and that their expression is regulated in a tissue-specific manner and independently of ethylene in carnation flowers during senescence.

Key words: Carnation, Dianthus caryophyllus, ethylene perception, ethylene-receptor mRNAs, flower senescence, petal wilting.

Introduction

Ethylene is a plant hormone, which regulates many aspects of plant growth and development, such as seed germination, fruit ripening and tissue senescence (Abeles et al., 1992). Ethylene also plays a crucial role in the senescence of carnation flowers (Manning, 1985; Peiser, 1986; Woodson et al., 1992). In carnation flowers, ethylene is first produced from the pistil and the evolved ethylene induces the autocatalytic ethylene production in petals, resulting in wilting of the petals, during the natural senescence of carnation flowers (ten Have and Woltering, 1997; Shibuya et al., 2000).

Previous studies on ethylene perception in plant tissues revealed the genes for ethylene receptors in diverse plant species, such as Arabidopsis (Chang et al., 1993; Hua et al., 1995), Rumex (Vriezen et al., 1997), melon (Sato-Nara et al., 1999), and so on. Now it is known that ethylene receptor genes make a family in plants; such as five members in Arabidopsis (ETR1, Chang et al., 1993; ERS1, Hua et al., 1995; ETR2, Sakai et al., 1998; EIN4, ERS2, Hua et al., 1998) and six members in tomato (Nr, Wilkinson et al., 1995; LeETR1, LeETR2, Zhou et al., 1996a, b; Lashbrook et al., 1998; LeETR4, LeETR5, Tieman and Klee, 1999; LeETR6, Imanishi et al., 2001).

So far, three putative ethylene receptor genes, DC-ERS1 (Chang et al., 1997), DC-ERS2 (Shibuya et al., 1998) and DC-ETR1 (Nagata et al., 2000) have been identified in carnation plants, indicating that carnation plants also have a family of ethylene receptor genes. However, little is known about the involvement of receptor genes in ethylene perception of carnation flowers, especially of petals, during senescence. In the
present study, changes in the mRNA levels for these three genes in the respective flower tissues were investigated by Northern blot analysis to assess their possible involvement in ethylene perception during senescence of carnation flowers.

Materials and methods

Plant materials
Carnation (Dianthus caryophyllus L. cv. Reiko) flowers at the pre- and full-opening stages were obtained from a local grower. The flower just fully opened was designated as the flower on day 0. Petals, ovaries, styles, leaves, and stems were detached from flowers after the assay for ethylene production, immediately frozen in liquid N2 and stored at −80 °C until isolation of RNA.

Treatments of flowers
Carnation flowers were used at their full-opening stage (day 0). Flowers were trimmed to 5 cm in stem length and placed with their stem end in 20 ml water in 50 ml glass vials (one flower per vial). They were incubated at 23 °C under white fluorescent light (12 μmol m−2 s−1, 12/12 h light/dark) for 7 d for natural senescence. At 1 d intervals, three flowers were randomly taken for assay of ethylene production (as described below).

For ethylene treatment, the vials with flowers on day 0 were incubated in a 53.2 1 glass chamber with ethylene at 10 μl l−1 for 0–24 h under the same conditions as above but under continuous light. Samples of three flowers were collected at 4 h intervals from the glass chamber. To diffuse out the accumulated ethylene from flower tissues, the flowers were held in open air for 1 h prior to measurement of ethylene production. Then ethylene production was assayed and petals sampled as above.

For treatments with 1,1-dimethyl-4-(phenylsulphonyl) semicarbazide (DPSS), the stem end of carnation flowers was placed for 24 h from day 0 to day 1 in 20 ml of 0.2 mM DPSS solution in 50 ml glass vials. Then the flowers were transferred to distilled water and left under the same conditions as above. Ethylene production was measured and flower tissues were sampled as above.

Ethylene production assay
Ethylene production was determined by enclosing flowers in 350 ml glass containers (one flower per container) for 1 h at 23 °C (Kosugi et al., 1997). A 1 ml gas sample was taken into a hypodermic syringe from the inside of the container through a rubber septum of a sampling port on the container, and analysed for ethylene with a gas-chromatograph (Model 263–30, Hitachi) equipped with an alumina column and a flame ionization detector.

Construction of gene-specific probes DC-ERS1 and DC-ERS2 mRNAs and probes for DC-ETR1 and actin (DC-ACT1) mRNAs
Gene-specific probes for DC-ERS1 and DC-ERS2 were constructed from PCR products corresponding to the 3′-end sequence, ranging from the coding region to the 3′ untranslated region of these cDNAs. A partial-length cDNA for DC-ERS1 was amplified by RT-PCR with appropriate primers and total RNA from carnation leaves. PCR products were separated on a 1.0% (w/v) agarose gel and the putative band was recovered. The cDNA fragment was ligated into pBluescript II SK (+) (Stratagene), and the resultant plasmid pDC-ERS1-3′ was amplified in E. coli XL-1 Blue (Stratagene). pDC-ERS1-3′ was sequenced and revealed to be identical to the corresponding region of DC-ERS1. The plasmids pDC-ERS1-3′ and pDC-ERS2 (Shibuya et al., 1998) were used as templates for amplification of DES1-3′UTR and DES2-3′UTR, respectively. The upstream and downstream primers were, 5′-GACA-AATTAGTTTCAGGT-3′ and 5′-GGGTAGACCTGTAATAAAT-3′, respectively, for the DC-ERS1-specific probe (DES1-3′UTR), and 5′-GGGAATTCCTTTGGATTGA-GAGT-3′ and 5′-CCCTCAGACTTCTAGATTITG-3′, respectively, for the DC-ERS2-specific probe (DES2-3′UTR). The DES1-3′UTR probe corresponds to positions 1765 bp to 1976 bp of DC-ERS1, and the DES2-3′UTR probe to positions 1675 bp to 2169 bp of DC-ERS2 (Fig. 1). The cDNAs were labelled with 32P-dCTP by random priming using Multiprime DNA labelling systems (Amersham Pharmacia Biotech) according to the manufacturer’s instructions and used as probes in the Northern blot analysis. The specificity of the gene-specific probes was checked by testing cross hybridization between the probes and genomic DNA fragments (Fig. 2A).

For construction of the probe for DC-ETR1 (DET1-P), a fragment of entire length was cut out from the plasmid including the 897 bp long partial length cDNA of DC-ETR1 (pWS6, Nagata et al., 2000) with appropriate enzymes (Fig. 1). The cDNA fragment was labelled with 32P as above. This probe’s specificity was confirmed by differences in the size of mRNAs detected by DET1-P and DES2-3′UTR (Fig. 2B). The construction of the 32P-labelled DNA probe for mRNA of carnation actin (DC-ACT1) was described previously (Waki et al., 2001).

Northern blot analysis
Total RNA was isolated by the SDS-phenol method (Palmiter, 1974) from petals, ovaries, styles, leaves or stems of carnation plants, and purified to poly(A)1+ RNAs using Oligotex-dT30 (Takara) according to the manufacturer’s instructions. Northern blot analysis was conducted with the poly(A)1+ RNA as described previously (Shibuya et al., 2000). Blots were used for multiple hybridization after stripping in boiling 0.1% (w/v) SDS. Hybridization signals for DC-ERS2 and DC-ETR1 mRNAs were normalized using the NIH image software against the signals of actin mRNA, and data obtained were shown as the relative levels of mRNAs.

Southern blot analysis
Genomic DNA was isolated from carnation petals by the CTAB method (Murray and Thompson, 1980). Ten μg of the DNA was digested with EcoRI or HindIII, separated on a 0.8% (w/v) agarose gel and transferred to nylon membrane filters (Hybond N+, Amersham Pharmacia Biotech). Hybridization was conducted as mentioned above.

Results

Specificity of the probes
When the present study was started, only two full-length cDNAs for ethylene receptor genes, DC-ERS1
(Chang et al., 1997) and DC-ERS2 (Shibuya et al., 1998), had been identified in carnation plants. Therefore, to detect mRNAs for DC-ERS1 and DC-ERS2 separately by Northern blot analysis, gene-specific probes with the 3' periphery region of cDNAs for the respective genes were constructed, and called DES1-3'UTR and DES2-3'UTR, respectively (Fig. 1). Figure 2A shows a profile of Southern blot analysis with these two probes and EcoRI- or HindIII-digests of genomic DNA of carnation plants. Each probe detected only one band that migrated to different positions in respective lanes, indicating the specificity of the probes to corresponding genes, DC-ERS1 and DC-ERS2. It is reasonable to expect that each probe is also specific to mRNA corresponding to its parent gene. These results suggested that there is one gene each for these genes in the carnation genome. In practice, by Northern blot analysis with mRNAs obtained from flower tissues of carnation, DES2-3'UTR detected only one mRNA of 2.3 kbp, whereas DES1-3'UTR did not detect any mRNA, indicating that the amount of DC-ERS1 mRNA was below the limit of detection in carnation flower tissues.

After the construction of DES1-3'UTR and DES2-3'UTR probes was completed, Nagata et al. cloned the cDNA of the third gene for the ethylene receptor, DC-ETR1, from carnation plants (Nagata et al., 2000). This cDNA was obtained as a partial-length cDNA of 897 bp and is considered to belong to the ETR-subgroup of ethylene receptors judging from its deduced amino acid sequence. Although the cloning of full-length cDNA of DC-ETR1 and its analysis was being conducted by other researchers and would be reported elsewhere, the authors wished to include preliminary findings of DC-ETR1 in the present work on DC-ERS1 and DC-ERS2. Therefore, using the cDNA as template the probe of 897 bp, DET1-P, was constructed for the detection of DC-ETR1 mRNA. Comparison of the efficacy of DET1-P with that of DES2-3'UTR, revealed that the former detected the mRNA of 2.6 kbp, whereas the latter detected the mRNA of 2.3 kbp (Fig. 2B). These results indicated that DES2-3'UTR does not cross-hybridize with DC-ETR1 mRNA, and DET1-P does not cross-hybridize with DC-ERS2 mRNA. It should be noted that the DET1-P probe detected only one mRNA in total RNA extracted from petals at the full-opening stage, indicating its specificity to DC-ETR1 mRNA. However, this does not always rule out the possible hybridization with mRNAs for unidentified ethylene receptors, if any, other than DC-ETR1 in carnation plants since the probe was derived from the coding region of DC-ETR1 cDNA. The difference in size of mRNAs detected by DET1-P or DES2-3'UTR may be related to the probable difference in molecular mass of the translation product between DC-ETR1 and DC-ERS2; the former is an ETR-type receptor and has a response regulator domain.
Changes in mRNA levels of ethylene receptors during natural senescence of carnation flowers

Figure 3 compares the changes in ethylene production in whole flowers and in levels of mRNA for DC-ERS2 and DC-ETR1 in petals, ovaries and styles of the flowers during opening and subsequent senescence. The photographs for DC-ERS1 were omitted from the figure since the mRNA for DC-ERS1 was not detected in any of the RNA samples. Ethylene production, which was monitored to assess the progress of senescence in the flowers, was detected on day 4 after full-opening (day 0) of the flowers, attaining the maximum rate on day 6 (Fig. 3A).

In petals, the mRNA for DC-ERS2 was detected at the pre-opening stage (day –2) and their levels increased on days 0 and 1, then showed a decreasing trend toward the late stage of senescence (Fig. 3B, C). The change in the level of DC-ETR1 mRNA was similar to, but less than, that of DC-ERS2 mRNA. In Fig. 4, the results for
DC-ERS2 were given by the means of three experiments; one shown in Fig. 3 and two additional separate experiments. The levels of DC-ERS2 mRNA on day 4 and day 6, when ethylene production started and reached the maximum, were 46% and 16%, respectively, of the level on day 0 (Fig. 4).

In ovaries and styles, the mRNAs for DC-ERS2 and DC-ETR1 were also detected at the pre-opening stage. The level of DC-ERS2 mRNA in ovaries increased a little and that of DC-ETR1 remained almost constant during senescence of the flowers (Fig. 3B, C). The levels of DC-ERS2 and DC-ETR1 mRNAs in styles were almost unchanged.

**Effects of exogenous ethylene on mRNA levels of ethylene receptors in carnation petals**

Carnation flowers on day 0 were exposed to 10 μl l⁻¹ ethylene for 0–24 h. At 4 h intervals, ethylene production in whole flowers and mRNA levels of ethylene receptors in petals were determined (Fig. 5A). Ethylene production was induced in carnation flowers 4 h after the start of exposure to ethylene; the ethylene production rate increased to a maximum at 20 h, and declined thereafter (Fig. 5A). The mRNA levels of DC-ERS2 and DC-ETR1 increased transiently at 4 h, then remained at the level at 0 h (Fig. 5B, C). DC-ERS1 mRNA was not detected in petals during exposure to ethylene for 24 h.

**Effect of DPSS on mRNA levels of ethylene receptors in carnation petals**

DPSS is an anti-senescence preservative for cut carnation flowers, which completely inhibits ethylene production in the flowers and lengthens their vase-life by about 2-fold as compared with that of non-treated flowers (Midoh et al., 1996). The mechanism of action of DPSS has not been fully elucidated yet, although Onoue et al. recently showed that DPSS suppressed ethylene production through inhibition of the transient increase in ABA content in tissues of the flowers (Onoue et al., 2000).

The effect of DPSS on the level of mRNAs for ethylene receptors was investigated. Carnation flowers treated with 0.2 mM DPSS kept their vase-life until day 13 after the
full-opening of the flowers, and no ethylene production was detected during the incubation period. The DPSS-treated carnation flowers lost their vase-life with desiccation starting from the petal periphery, which is typical for ethylene-independent deterioration of the flowers after long storage. The level of \textit{DC-ERS2} mRNA in petals decreased gradually toward the end of the 13 d incubation period, reaching 30\% of the mRNA on day 0 (Fig. 6). The level of \textit{DC-ETR1} mRNA decreased similarly (data not shown). \textit{DC-ERS1} mRNA was not detected during the incubation period of 13 d. It should be noted that the decrease in the mRNA levels of \textit{DC-ERS2} and \textit{DC-ETR1} in petals occurred in the absence of endogenous production of ethylene, and DPSS treatment lowered the rate of decrease in the mRNA level by 2-fold as compared with that found in naturally senescing petals (Fig. 3).

\textbf{Presence of mRNAs for ethylene receptors in leaves and stems of carnation plants}

For reference, the mRNAs for \textit{DC-ERS1}, \textit{DC-ERS2} and \textit{DC-ETR1} were examined to find out whether they were present in tissues other than the flower of carnation plants. Northern blot analysis revealed that the mRNAs for \textit{DC-ERS2} and \textit{DC-ETR1} were present in carnation leaves and stems at the full-opening stage. The mRNA levels of both genes were higher in the leaf tissue than others. \textit{DC-ERS1} mRNA was not detected in the leaf or stem tissues either (Fig. 7).

\textbf{Discussion}

Exogenous ethylene induces autocatalytic ethylene production and wilting in petals of carnation flowers. Furthermore, it was recently shown that ethylene generated in the gynoecium acts as a diffusible signal and triggers the onset of ethylene production in the petals in natural senescence (ten Have and Woltering, 1997; Shibuya \textit{et al}., 2000) and in pollination–induced senescence (Jones and Woodson, 1999) of the flowers. Thus carnation flowers are thought to be an excellent model system for the study of ethylene perception and its signalling. Bearing this in mind, Northern blot analysis was performed to search for genes for ethylene receptors acting in carnation flowers during senescence.

Three genes encoding ethylene receptors have been isolated from carnation plants; full-length cDNAs for \textit{DC-ERS1} (Charng \textit{et al}., 1997) and \textit{DC-ERS2}
Recently, ethylene receptors have been shown to act as negative regulators of ethylene responses in *Arabidopsis* and tomato (Hua and Meyerowitz, 1998; Tieman et al., 2000; Ciardi et al., 2000). That is, loss-of-function mutation in four receptor genes (*ETR1, ETR2, EIN4 and ERS2*) resulted in strong constitutive ethylene responses in *Arabidopsis* (Hua and Meyerowitz, 1998). In a transgenic tomato plant, reduced expression of one receptor gene, *LeETR4*, resulted in constitutive ethylene responses such as leaf epinasty and accelerated flower senescence (Tieman et al., 2000). Furthermore, the transgenic tomato plants over-expressing *Nt* gene showed an ethylene-insensitive phenotype (Ciardi et al., 2000). These results indicate that there is an inverse relationship between the level of ethylene receptors and the sensitivity to ethylene and that reduction in the amount of ethylene receptor proteins increases ethylene sensitivity of plant tissues. According to these notions, the decrease in the level of *DC-ERS2* mRNA (and probably the level of *DC-ERS2* protein) in petals may cause an increase in sensitivity to ethylene and, hence, an accelerated petal wilting in carnation flowers during senescence. However, to address the relationship between the level of ethylene receptors and the sensitivity to ethylene, which determines flower longevity in carnation flowers, further studies are needed to determine the content of ethylene receptor proteins as well as their threshold levels to exert responses to ethylene in flower tissues.

By contrast, Muller *et al.* recently reported a parallel relationship between the level of ethylene receptor transcripts and the flower longevity, which is regulated by sensitivity to ethylene in rose flowers (Muller  et al., 2000). They found that the expression of *RhETR3*, one of four genes identified in rose, increased in senescing flowers of ‘Bronze’, a cultivar with a short floral life, while it remained at low levels in ‘Vanilla’, a cultivar with a long floral life. Furthermore, they found that the expression of the gene was increased by ABA and ethylene treatment, which stimulated the senescence of the rose flowers. From these results, they proposed that the differences in flower life among rose cultivars could be due to differences in receptor levels.

References


