Abstract

Using a combination of approaches, three EIN3-like (EIL) genes DC-EIL1/2 (AY728191), DC-EIL3 (AY728192), and DC-EIL4 (AY728193) were isolated from carnation (Dianthus caryophyllus) petals. DC-EIL1/2 deduced amino acid sequence shares 98% identity with the previously cloned and characterized carnation DC-EIL1 (AF261654), 62% identity with DC-EIL3, and 60% identity with DC-EIL4. DC-EIL3 deduced amino acid sequence shares 100% identity with a previously cloned carnation gene fragment, Dc106 (CF259543), 61% identity with Dianthus caryophyllus DC-EIL1 (AF261654), and 59% identity with DC-EIL4. DC-EIL4 shared 60% identity with DC-EIL1 (AF261654). Expression analyses performed on vegetative and flower tissues (petals, ovaries, and styles) during growth and development and senescence (natural and ethylene-induced) indicated that the mRNA accumulation of the DC-EIL family of genes in carnation is regulated developmentally and by ethylene. DC-EIL3 mRNA showed significant accumulation upon ethylene exposure, during flower development, and upon pollination in petals and styles. Interestingly, decreasing levels of DC-EIL3 mRNA were found in wounded leaves and ovaries of senescing flowers whenever ethylene levels increased. Flowers treated with sucrose showed a 2 d delay in the accumulation of DC-EIL3 transcripts when compared with control flowers. These observations suggest an important role for DC-EIL3 during growth and development. Changes in DC-EIL1/2 and DC-EIL4 mRNA levels during flower development, and upon ethylene exposure and pollination were very similar. mRNA levels of the DC-EILs in styles of pollinated flowers showed a positive correlation with ethylene production after pollination. The cloning and characterization of the EIN3-like genes in the present study showed their transcriptional regulation not previously observed for EILs.

Key words: Carnation, Dianthus, EIN3, ethylene, flower senescence, pollination, signal transduction, wounding.

Introduction

Ethylene, a gaseous plant hormone, plays an important role in plant growth and development (Abeles et al., 1992). Flower senescence is one of the developmental processes in which ethylene plays a key role (Reid and Wu, 1992). In many commercially valuable crops flower senescence is associated with increased production of ethylene. The climacteric rise of endogenous ethylene in these flowers has been shown to play a regulatory role in the events leading to the death of some of the flower organs. Ethylene perception is an essential requirement for the initiation and progression of the senescence programme (Borochov and Woodson, 1989). The response of flower petals to ethylene depends on the physiological age and developmental stage of the tissue at the time of exposure. It is thought that the increase in ethylene responsiveness during petal development culminates in the ethylene climacteric. Therefore, changes in ethylene responsiveness during development, most likely mediated by changes in ethylene signalling, are a critical part in the developmental sequence of events that
lead to flower senescence (Woodson et al., 1992; Verlinden et al., 2002).

The characterization of a limited number of putative ethylene receptors in carnations, DC-ERS1, DC-ERS2, and DC-ETR1, indicates that, so far, changes in ethylene responsiveness during petal development cannot be explained by changes in mRNA accumulation of ethylene receptor genes (Shibuya et al., 2002). In addition, ethylene binding does not change throughout petal development suggesting that other steps in ethylene signalling may control ethylene responsiveness (Brown et al., 1986). Using the ethylene triple response phenotype to screen for mutants that are defective in ethylene responses, a number of ethylene signalling components besides ethylene receptors have been studied in the model plant Arabidopsis thaliana, including CTR1, EIN2, EIN3, EIN5, EIN6, and EER1 (Wang et al., 2002). Their positions in the ethylene signal transduction cascade have been determined. In the case of CTR1, EIN2, and EIN3 a putative function has been assigned. One of the downstream components, EIN3, has received more attention than any other. EIN3, located downstream of EIN2 in the ethylene signal pathway, is a novel nuclear localized protein that belongs to a multigene family of transcription factors in Arabidopsis. There are six putative members of this family in Arabidopsis. Of these, EIN3, EIL1, and EIL2 can rescue the ein3 mutant (Wang et al., 2002). Genes corresponding to EIN3 or EILs have also been cloned from other plant species including the tobacco TEIL (NtEIL1) (Kosugi and Ohashi, 2000), NtEIL2, NtEIL3, NtEIL4, and NtEIL5 genes (Rieu et al., 2003), the tomato LeEIL1, LeEIL2, LeEIL3 (Tieman et al., 2001), and LeEIL4 genes (Yokotani et al., 2003), the carnation DC-EIL1 gene (Waki et al., 2001), and two mung bean VR-EIL genes (Lee and Kim, 2003).

So far, EIN3/EIL mRNA accumulation levels have not been found to be affected by ethylene treatment or shown significant changes throughout plant development. Furthermore, overexpression of several EIN3/EIL members confers constitutive ethylene responses without significant changes in transcript levels. These findings led to the hypothesis that EIN3 may be regulated at the protein level (Wang et al., 2002). Indeed, this has been confirmed and discussed recently in a number of articles (Guo and Ecker, 2003; Yanagisawa et al., 2003; Potuschak et al., 2003; Maniatis et al., 1982).

Yanagisawa et al. (2003) found that glucose enhances the degradation of EIN3, a process mediated by the glucose sensor hexokinase, and that ethylene enhances the stability of EIN3. As de novo protein synthesis is not required for ethylene-mediated EIN3 protein accumulation, ethylene represses constitutive degradation of EIN3, hence activating downstream gene expression (Guo and Ecker, 2003). In Arabidopsis, EIN3 is targeted for degradation by the ubiquitin/proteasome pathway, being specifically recognized by the F-box proteins EBF1/EBF2 (Guo and Ecker, 2003). In contrast to some other targets in SCF-dependent proteolysis, EIN3 does not need a post-translational phosphorylation in order to be recognized (Potuschak et al., 2003).

Although the work described above in Arabidopsis supports post-transcriptional regulation of EIN3, the cloning and characterization of EIN3 homologous genes and studies of their mRNA accumulation in other species can give an insight into the changes in ethylene responsiveness observed in those species. The intention was to clone and characterize EIN3 homologous genes in carnation to see if transcriptional regulation is present and furthermore to see if mRNA levels can be correlated with changes in ethylene responsiveness and production.

Materials and methods

Plant material
Carnations (Dianthus caryophyllus L. cv. Improved White Sim) were grown under standard greenhouse conditions (21 °C day/night, 60–80% relative humidity) in a peat–perlite substrate. Plants were watered as needed and fertilized once a week with a 400 mg l⁻¹ N based on a 20N:10P:20K fertilizer (Plant Products Co. Ltd., Ontario, Canada).

Cloning of EIN3 homologues
First strand cDNA was generated according to standard protocols (Maniatis et al., 1982) from mRNA extracted from senescent carnation petals. PCR was performed with degenerate oligonucleotides based on the conserved EIN3 domain. The forward and reverse primers used for PCR were: 5'-AGT GAR GTN TGY AAR GCN CAR-3' and 5'-YTC YTT NGC NGT CAT YTT RTC YTG-3', respectively. A 530 bp PCR product was cloned into pCR 2.1 TOPO cloning vector using a TA cloning kit (Invitrogen, Carlsbad, CA). Subsequent sequencing of the vectors containing these fragments revealed the cloning of three distinct EIN3-like genes: DC-EIL1/2, DC-EIL3, and DC-EIL4. Full-length cDNAs were generated using an Invitrogen Gene Racer kit that includes tools to perform both 5'-RACE and 3'-RACE (Invitrogen, Carlsbad, CA). Sequencing was performed by Davis Sequencing Inc. (Davis, CA). Phylogenetic analysis was carried out with the Clustal W program (Thompson et al., 1994).

RNA extraction and northern blot analysis
Total RNA was extracted according to the previously described protocols (Woodson and Lawton, 1988). Briefly, 2 g of frozen tissue was powdered under liquid nitrogen and homogenized in equal volumes (10 ml) of phenol:chloroform:isoamylalcohol (25:24:1, by vol.) and an extraction buffer containing 50 mM TRIS–HCl (pH 8.0), 5% phenol (pH 8.0), 6% sodium p-aminosalicylate, and 1% (v/v) β-mercaptoethanol. Following phase separation, nucleic acids were precipitated by the addition of 1/20 vol. of 4 M sodium acetate (pH 6.0) and 2 vols of ethanol overnight at −20 °C. The precipitate was resuspended in diethylpyrocarbonate (DEPC)-treated water, reprecipitated by the addition of 3 vols of 4 M sodium acetate (pH 6.0) and incubated on ice for 1.5 h. The precipitated RNA was dissolved in DEPC-treated water. RNA content was determined spectrophotometrically. Northern blot analysis was performed according to standard protocols (Jones and Woodson, 1999).

Construction of gene-specific probes and Southern blot hybridization
Gene-specific probes were constructed from the 3' ends of the three genes. Plasmids containing the 3' ends of the three genes described above were used as template in PCR amplification of the probes.
The primers used for amplification were as follows: DC-EIL1/2 forward: 5′-CGGTTTCCCAAGGAATGTTCTGG-3′ and DC-EIL1/2 reverse: 5′-ACTAGTCTATATGATGGGCCACT-3′; DC-EIL3 forward: 5′-CGGTTTGGTTCTAGTGAGCA-3′ and DC-EIL3 reverse: 5′-TCTGTACCAAGCCTGATGTTGCC-3′; DC-EIL4 forward: 5′-AAACGGGTTCGCGGTGGCTT-3′ and DC-EIL4 reverse: 5′-TCAGTTTTCGCCACTGGGACA-3′. pCR 2.1 plasmids containing the 3′ half of DC-EILs cDNA were separated on a 1% agarose gel, and then transferred to a nylon membrane. Southern blot analysis was performed according to standard procedures (Maniatis et al., 1982).

Wounding treatment

 Mature leaves were detached from the carnation plants, washed, and placed in distilled water for 24 h. Subsequently, leaves were weighed, wounded with a wire brush on both sides, and placed in 50 ml beakers containing 25 ml distilled water at room temperature. For each time point, four leaves were used. One h prior to the time of ethylene measurement the wounded leaves were enclosed in a 50 ml vial containing 2 ml distilled water and incubated at room temperature. A 1 ml gas sample was collected with a hypodermic syringe through the rubber septum of the vial and analysed for ethylene with a gas chromatograph (Varian CP-3800, Varian Inc., Palo Alto, California). Separate tissue was collected and frozen at 0, 0.5, 1, 2, 4, 6, and 12 h after wounding, and stored for later RNA extraction.

Flower development

Eight to ten carnation flowers were harvested at the following stages of development: closed bud, opening bud, paintbrush, anthesis (petals reflexed), open, early senescent, mid-senescent, and late senescent stage of flower development. Petals, ovaries, and styles were separated and frozen for later RNA extraction. Ethylene production of the flowers was determined by enclosing 1 g of petals from each time point in a 50 ml vial with a rubber septum and incubating them for 15 min at room temperature. A 1 ml gas sample was collected with a hypodermic syringe through the septum and analysed for ethylene with a gas chromatograph (Varian CP-3800, Varian Inc., Palo Alto, California).

Ethylene treatment

Carnation flowers were harvested at anthesis and their stems re-cut to 25 cm, placed in flasks with distilled water, and subsequently treated with 10 μl l⁻¹ ethylene for 0, 1, 2, 4, 6, 12, and 24 h. Petals, styles and ovaries from 8–10 flowers were collected at each time point, immediately frozen in liquid nitrogen, and stored at −80 °C for later RNA extraction.

Sucrose treatment

Carnation flowers harvested at anthesis, re-cut to 25 cm in length, were placed in 1.0 l flasks containing distilled water or a 5% sucrose solution and held in controlled environmental conditions (24/18 °C day/night temperature, 100 μE m⁻² s⁻¹). Solutions were changed every day. Petals and ovaries were collected at 0, 2, 4, and 6 d, and frozen for later RNA extraction.

Pollination

RNA from styles of pollinated flowers was obtained from Dr Michelle Jones (The Ohio State University). Treatment and RNA extraction of this tissue is described in Jones and Woodson (1997).

Results

Three full-length EIN3-like cDNAs were cloned from carnation: DC-EIL1/2 (AY728191), DC-EIL3 (AY728192), and DC-EIL4 (AY728193). The deduced amino acid sequences of the three genes share 65%, 54%, and 54% identity, respectively, with A. thaliana EIN3 (NM_112698.2). The DC-EIL1/2 deduced amino acid sequence shares 98% identity with the previously cloned D. caryophyllus DC-EIL1 (AF261654) (Waki et al., 2001), 62% identity with DC-EIL3, and 60% identity with DC-EIL4. Following genomic DNA analysis (data not shown), it was concluded that DC-EIL1 and DC-EIL1/2 are derived from the same gene in carnation and that the differences in the nucleotide sequences are most likely due to the use of different carnation cultivars. Although the two genes are extremely similar, it was decided to name the gene DC-EIL1/2 (see Discussion). The DC-EIL3 deduced amino acid sequence shares 100% identity with a previously cloned carnation gene fragment, Dc106 (CF259543), 61% identity with DC-EIL1 (AF261654), and 59% identity with DC-EIL4. DC-EIL4 shared 60% identity with DC-EIL1 (AF261654). All DC-EILs contain the conserved EIN3 domain (Chao et al., 1997).

Phylogenetic analysis of the EIN3 homologues (Fig. 1) described to date, revealed the presence of three phylogenetic clusters (Thompson et al., 1994). DC-EIL1/2 is located in one cluster, which contains the Arabidopsis EIL1 and EIN3 genes. DC-EIL3 and DC-EIL4 belong to the same subcluster as the Arabidopsis EIL2 and EIL3 genes. DC-EIL3, most closely related to Arabidopsis EIL2, however, is defined by its own slightly divergent subcluster.

In order to verify the specificity of the probes used for the northern blot hybridization, the probes were hybridized with the 3′ region of the DC-EIL1/2–4 cDNAs. Each probe detected only one band, indicating that the probes are gene specific (Fig. 2).

Ethylene production by wounded leaves increased after 1 h, peaked at 2 h, and returned to the basal level after 4 h of wounding (Fig. 3A). Upon wounding treatment no changes in DC-EIL1/2 and DC-EIL4 mRNA levels were observed. However, DC-EIL3 showed a temporary decrease in mRNA accumulation at 1 h and 2 h after wounding (Fig. 3B) which was negatively correlated with a temporary increase in ethylene production (Fig. 3A).

Ethylene measurement of petals of the different developmental stages of carnation flowers used in this study showed a typical ethylene climacteric (Fig. 4A). Throughout flower development DC-EIL3 and DC-EIL4 mRNA accumulated in petals up to the late senescent and mid-senescent stages of development, respectively (Fig. 4B). In styles, however, it was mainly DC-EIL1/2 and DC-EIL3 which accumulated through the late senescent stage of flower development. In ovaries, after a small increase up to the stage of open bud, DC-EIL1/2 and DC-EIL3 mRNA accumulation decreased slightly until the open flower stage of development, and then decreased considerably thereafter. DC-EIL3 mRNA levels, in particular, dropped rapidly in early senescent flowers when the petals are...
starting to produce ethylene. Limited changes were observed in the mRNA levels of DC-EIL4, although a slight decrease in ovaries and some up-regulation in petals after anthesis were noticed (Fig. 4B).

Treatment of flowers with ethylene for 24 h revealed that, in petals and styles, after an initial rapid increase, DC-EIL1/2 and DC-EIL4 mRNA levels gradually declined from 1 h after the start until the end of the ethylene treatment (Fig. 5). DC-EIL3 mRNA accumulated slower in petals than DC-EIL1/2 and DC-EIL4 transcripts upon ethylene treatment and reached their highest levels after 4 h. DC-EIL3 mRNA levels remained elevated from 4 h to 12 h, and afterward decreased slightly. In ovaries, a decrease in DC-EIL3 mRNA levels was observed after 4 h of ethylene treatment. No notable changes were found in the levels of DC-EIL1/2 and DC-EIL4 mRNAs in the ovaries during ethylene exposure.

Following treatment of flowers with 5% sucrose, a 2 d delay in DC-EIL3 transcript accumulation in petals was observed when compared with the control (Fig. 6). In contrast to the changes in DC-EIL3 mRNA levels in control and sucrose-treated petals, DC-EIL1/2 and DC-EIL4 mRNA levels decreased over time. However, the decrease in mRNA levels of both DC-EIL1/2 and DC-EIL4 was slower in sucrose-treated than the control petals. In the ovaries, lower DC-EIL1/2 and DC-EIL3 mRNA levels were observed in control tissues than in the comparable sucrose-treated ovaries.

After pollination, the mRNA accumulation in styles of DC-EIL1/2 and DC-EIL4 showed a peak at 6 h after pollination, decreased at 12 h to low levels, and subsequently increased to peak again at 36 h after pollination (Fig. 7). Although the pattern of mRNA abundance in styles for the two genes was similar, DC-EIL1/2 showed higher levels of accumulation than DC-EIL4. DC-EIL3 accumulation also peaked at 6 h and 36 h after pollination, but the decrease at 12 h observed for DC-EIL1/2 and DC-EIL4 was not as noticeable or slightly delayed. Flowers treated with norbornadiene (NBD) showed higher mRNA levels of DC-EIL1/2 and DC-EIL4 in styles than transcript levels observed in the pollinated styles at the same time points. However, DC-EIL3 mRNA accumulation in the styles of pollinated flowers was seemingly unaffected by the NBD treatment.
Discussion

Flower senescence is associated with increased ethylene production in many flowers (Borochov and Woodson, 1989). Ethylene responsiveness in carnation petals increases greatly during development up to the onset of senescence (Lawton et al., 1990; Verlinden et al., 2002). In this study, three EIN3 homologous genes, DC-EIL1/2, DC-EIL3, and DC-EIL4, were cloned and characterized, to understand the changes in ethylene responsiveness during flower development and senescence that lead to the ethylene climacteric.

When compared with other EILs described to date from different organisms, the carnation DC-EILs show high similarity within the EIN3 conserved domain suggesting that they are indeed functional EIN3-like genes (Chao et al., 1997). A previously cloned and characterized EIN3 homologous gene from carnation, DC-EIL1, is very similar to DC-EIL1/2 (Waki et al., 2001). However, they are not identical. The DC-EIL2 cDNA has a longer 5′-end untranslated region. In addition, a 14 bp region where the two
cDNAs are different was found within the EIN3 conserved domain. This region translates into IRLPG (DC-EIL1) instead of EWWPQ (DC-EIL1/2). Furthermore, one point mutation in the 5' region before the EIN3 domain, three point mutations within the EIN3 domain, and 11 point mutations in the 3' region outside the EIN3 domain were observed. Since the 14 bp region that is different is part of the conserved EIN3 domain and may result in functional differences from DC-EIL1, as well as the fact that DC-EIL1 and DC-EIL2 were isolated from different cultivars of carnation, it was elected to name this gene DC-EIL1/2.

By contrast with findings in other organisms (Wang et al., 2002), at least one of the EIN3-like genes cloned in this study, DC-EIL3, if not all of the DC-EILs described here, appear to be regulated by ethylene at the transcriptional level. Its mRNA levels in petals and styles increased not only after ethylene treatment but also throughout normal flower development, a pattern that has also been observed for some senescence-related genes (Lawton et al., 1989; Jones et al., 1995; Verlinden et al., 2002). This pattern of mRNA accumulation would also be expected of a gene regulating ethylene responsiveness and therefore timing of petal senescence. In the course of natural flower senescence, SR8 and SR12, two senescence–related gene transcripts start to accumulate in carnation flower petals at the onset of the ethylene climacteric (Lawton et al., 1989). In addition, the increase in ethylene responsiveness in carnation petals is associated with a concomitant increase in the abundance of senescence-related SR8 and SR12 mRNAs, when pre-senescent tissues are exposed to ethylene (Lawton et al., 1990; Verlinden et al., 2002). Ethylene responsive elements (EREs) were found in the promoter region of SR8 and SR12 genes (Verlinden et al., 2002). These data, together with the fact that EIN3-like proteins have been shown to interact with EREs (Solano et al., 1998), and the observation that DC-EIL3 mRNA accumulation closely mimics the increase in ethylene responsiveness during petal development suggest that DC-EIL3 may regulate SR8 and SR12 gene expression, and therefore
play an essential role in the senescence programme. Future studies demonstrating functionality through in vitro and in vivo experiments including transgenic plants should bear this out.

The temporary decrease in DC-EIL3 mRNA accumulation in leaves upon wounding seemed to be negatively correlated with ethylene production, indicating again that this gene may play an important role in ethylene signalling and the response of carnation plants to ethylene. DC-EIL3 mRNA levels could possibly be lowered at the time of peak ethylene production in order to suppress the onset of senescence in leaves by decreasing tissue sensitivity to ethylene. Dropping the levels of DC-EIL3 mRNA might also be a signal to activate some defence mechanisms of the plant, as has been seen in other gene expression studies (Lorenzo et al., 2003). In addition, when DC-EIL3 mRNA started accumulating in presenescing tissues (petals and styles) it decreased in ovaries. The mechanism involved in the response seen in wounded leaves, a temporary decrease in mRNA levels, correlated to ethylene production, may also be responsible for the decrease in mRNA observed in ovaries during senescence when significant amounts of ethylene evolve from the flower. This also suggests that regulation of DC-EIL3 in photosynthetically active tissue – the ovaries besides the sepals are the only green tissues of the flower – is different from tissues lacking significant photosynthetic capacity. However, further regulation including accumulation or degradation of DC-EIL3 at the protein level in leaves, ovaries, styles, and petals can again not be ruled out.

Sucrose and other sugars have been shown to modulate plant growth and development (Koch, 2004). For example, Ohto et al. (2001) reported that sucrose delays the flowering time in Arabidopsis. Sucrose has also been shown to decrease ethylene responsiveness in carnation petals (Verlinden and Garcia, 2004). Sugars are known to extend the vase-life of flowers (van Doorn, 2004), therefore the delay of the increase of DC-EIL3 mRNA accumulation in petals can be expected. However, the maintenance of high levels of DC-EIL3 mRNA in ovaries treated with sucrose was unexpected, but again points to differential regulation of mRNA accumulation in tissues capable of photosynthesis. The higher levels of DC-EIL1/2 and DC-EIL4 mRNA in the flowers treated with sucrose are contradictory to observations of decreased ethylene responsiveness and indicate that interactions between sugar and ethylene signalling must be tissue-dependent and complex.

Jones and Woodson (1997) studied ethylene production in carnation flowers after pollination. They showed an initial burst of ethylene 1 h after pollination (not inhibited by NBD), followed by a peak at 12 h, then a higher peak at 36 h after pollination. In the pollinated flowers treated with NBD, the 12 h peak was smaller than in the pollinated flowers kept in air. A positive correlation is shown here between ethylene production after pollination and accumulation of DC-EIL1/2 and DC-EIL4 mRNAs. In styles of pollinated flowers, DC-EIL1/2 and DC-EIL4 mRNA accumulated up to 6 h, then decreased to 12 h, and then rose again to another, smaller peak, at 36 h after pollination. Again, as seen in sucrose-treated tissues and ethylene treatment, DC-EIL3 showed different patterns of mRNA accumulation. Slower DC-EIL3 accumulation shifted its peak mRNA level from 6 h to 8 h after pollination similar to its slower response to ethylene treatment in styles. However, in the case of DC-EIL3 there was no clear difference in mRNA levels in pollinated flowers treated or not treated with NBD. Because NBD is an ethylene action inhibitor, it would be expected that DC-EILs mRNA levels would be lower in flowers treated with NBD. The fact that the mRNA levels are actually higher indicates that an overriding developmental mechanism may play a role in the accumulation of DC-EIL3 transcripts. DC-EIL1/2 mRNA accumulated to notably higher levels in flowers treated with NBD at 12 h and 48 h, a response that could be the result of a shift in the pattern of accumulation or developmental cues independent of ethylene action. Taken together, these observations point to the regulation of the pollination response through changes in mRNA accumulation of DC-EIL1/2–4.

In conclusion, the three EIN3-like genes in this study are regulated at the transcript level. At least one of these genes, DC-EIL3 shows significant regulation by ethylene. DC-EIL3 also showed developmental patterns of transcript accumulation that suggest a possible role for this gene in initiating and sustaining of the senescence programme of carnation petals. Complex patterns of accumulation of other DC-EIL transcripts have been observed indicating an intricate interplay between developmental and temporal factors, ethylene signalling and production, and growth and development in carnation.

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References


