Cloning and Characterization of a cDNA Encoding a Putative Nuclease Related to Petal Senescence in Carnation (*Dianthus caryophyllus* L.) Flowers

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Petal wilting, which involves the degradation of cell constituents, such as proteins, polysaccharides, lipids, and nucleic acids, is a determinant of the vase-life of carnation (*Dianthus caryophyllus* L.) flowers. In this study, a cDNA fragment, encoding a putative nuclease (*DcNUC1*), was isolated from carnation petals. In the petals of senescing carnation flowers, the level of the *DcNUC1* transcript increased, corresponding to the increase of ethylene production. It became maximal in wilted flowers, which suggested the association of *DcNUC1* expression with the wilting in carnation flowers.

**Key Words:** carnation, ethylene, flower senescence, nuclease, petal wilting.

**Introduction**

Petal (tepals) wilting is a key event determining the vase life of cut ornamental flowers. A highly orchestrated series of events, involved in the degradation of cell components, such as proteins, lipids, polysaccharides, and nucleic acids, and remobilization of the by-products for re-use, eventually lead to cell death. Morphological changes, typical to programmed cell death (PCD), such as chromatin condensation and nuclear fragmentation, have been detected in senescing petals of both ethylene-sensitive and -insensitive ornamental flowers (Orzaez and Granell, 1997; Yamada et al., 2001). DNA laddering (internucleosomal DNA cleavage), a widely used marker for apoptotic-like cell death in animal cells, has been detected during petal senescence in pea, petunia, freesia, Alstroemeria, gladiolus and gypsophila (Hoeberichts et al., 2005; Orzaez and Granell, 1997; Wagstaff et al., 2003; Xu and Hanson, 2000; Yamada et al., 2003). Furthermore, in daylily, a nuclease gene has been identified and shown to be expressed in senescing tepals (Panavas et al., 1999).

In carnation (*D. caryophyllus* L.) flowers, petal wilting is induced by endogenous or exogenously applied ethylene. In flowers that are undergoing natural senescence, ethylene is first produced in the pistil; the evolved ethylene initiates the autocatalytic ethylene production in petals, which in turn causes them to wilt (Shibuya et al., 2000; ten Have and Woltering, 1997). While petal wilting in senescing carnation flowers is probably associated with the degradation of DNA in petal cells, no studies on genes encoding nuclease in senescing carnation petals have been reported. Hence, in the present study, a cDNA fragment encoding nuclease (*DcNUC1*) was isolated from their fresh and senescing petals, and changes in their transcript levels were examined.

**Materials and Methods**

**Plant materials and treatment of flowers**

Carnation (*D. caryophyllus* L. ‘Nora’ and ‘Light Pink Barbara’ (‘LPB’)) flowers were obtained from a local grower. The day that petals of the outermost whorl became right angle to the pedicel (stem) was designated as day 0.

Stems of ‘LPB’ carnation flowers on day 0 were trimmed to 2 cm, and the cut ends were immersed in deionized water in 30 mL plastic vials (one flower per vial). They were incubated at 23°C under white fluorescent light emitting 50 µmol·m⁻²·s⁻¹ and under a 16 h photoperiod for 9 days for senescence to occur. Three flowers were randomly taken for daily assay of ethylene production. Ethylene production was determined by enclosing flowers in 350 mL glass containers (one flower per vial). They were incubated at 23°C under white fluorescent light emitting 50 µmol·m⁻²·s⁻¹ and under a 16 h photoperiod for 9 days for senescence to occur. Three flowers were randomly taken for daily assay of ethylene production. 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 withdrawn with a hypodermic syringe and injected into a gas chromatograph (GC-14, Shimadzu, Japan) equipped with an alumina column and flame ionization detector for ethylene determination. Immediately after the assay of ethylene production, petals were detached from 3 flowers and combined to make one sample, frozen in liquid N₂ and stored at −80°C until isolation of RNA.

**cDNA cloning**

Total RNA was isolated from petals of ‘Nora’ flowers,
at the stage of maximal ethylene production during senescence, by the cetyltrimethylammonium bromide (CTAB) method modified by Chang et al. (1993). A cDNA fragment (663 bp) was obtained by PCR by using the total RNA as an original template and the following two primers. The upstream primer was 5'-TCR GCS YTG TGY GTG TGG CCK GAY C-3' (nuclease multi F) and the downstream primer 5'-GGC YAG TCT NAC TCC WCC YTG NGC-3' (nuclease multi R). These primers were synthesized by referring to conserved nucleotide sequences among several nucleases of different origins (Perez-Amador et al., 2000). RT-PCR was performed according to the standard procedure with necessary optimization. The PCR product was cloned into pT7Blue (Novagen, USA) for sequencing. The cloned cDNA were sequenced for both strands by using ABI PRISM Big Dye Terminator v3.0 Cycle Sequence Kit, and ABI PRISM 310 sequencer (Applied Biosystems, USA). The cDNA lacked the 5' region for putative nuclease cDNA. Then, the upstream cDNA fragment (127 bp) was obtained by 5'-RACE (rapid amplification of cDNA ends; Frohman et al., 1988). The 5'-RACE was carried out by using the total RNA described above as the template and a sequence-specific primer with the 5'-RACE System Ver. 2 (Invitrogen, USA). The primer was derived from the nucleotide sequence of the above cDNA, 5'-GTG ATG GAG ATT CGA TTT GAG TTT-3'. Two cDNA fragments, obtained by RT-PCR and 5'-RACE, were reconstituted to make a composite cDNA (DcNUC1 cDNA, 790 bp).

Results and Discussion

We isolated a 790-bp long cDNA fragment that encoded a putative nuclease from ‘Nora’ carnation by using a combination of RT-PCR and 5'-RACE techniques. The cDNA, which encoded 263 amino-acid residues, corresponded to an almost entire open reading frame, but it lacked the 5'- and 3'-marginal sequences, as judged from the comparison of deduced amino-acid sequences among other nucleases of different origins (Fig. 1). The cDNA was designated as DcNUC1 (Dianthus garyophyllus nuclease 1, GenBank Accession No. AY986984).

**Southern blot analysis**

Genomic DNA was isolated from carnation petals by the CTAB method (Hasebe and Iwatsuki, 1990). Ten µg of genomic DNAs, obtained from ‘LPB’, was digested with BamH I, EcoR I, EcoR V, Hind III, Kpn I or Pvu II, and separated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, USA). A DcNUC1 cDNA fragment, 527 bp, was amplified by PCR and used as the probe for gel blot analysis. The DNA probe was labeled with DIG by using the DIG DNA labeling kit (Roche Applied Science, Japan). After hybridization with the blot, the hybridization signals were detected by chemiluminescence by using CSPD-Star (Roche Applied Science) as a substrate, and recorded on an X-ray film (RX-U, Fuji Photo Film, Japan).

**Northern blot analysis**

Total RNA was isolated from petals of ‘LPB’ carnation as described above. The probe for DcNUC1 transcript was the same as used for Southern blot analysis, but labeled with ECL (Amersham Pharmacia Biotech). Hybridization signals for DcNUC1 transcript were normalized by using NIH image software against the signals of rRNA.

**Fig. 1.** Amino acid sequence alignment of DcNUC1 and other nucleases. Residues that are identical in at least five of the sequences are shown as white characters in black boxes. Closed circles (●) represent residues involved in the binding of zinc atoms in nuclease P1. Diamonds (◇) depict residues involved in forming disulfide bonds in nuclease P1. The number sign (#) shows the structurally important glycosylation site in nuclease P1. Active sites for RNase and DNase activities in nucleases P1 and S1 are indicated by underlines. -,-, gaps to align the amino acid sequence. GenBank Accession Nos: Arabidopsis thaliana BFN1, U90264 (Perez-Amador et al., 2000); Aspergillus oryzae nuclease S1, P24021 (Iwamatsu et al., 1991); carnation DcNUC1, AY986984 (this study); daylily DAFSAG1, AF420010 (Hunter et al., 2002); daffodil DA6, AF083203 (Panavas et al., 1999); Penicillium citrinum nuclease P1, P24289 (Maekawa et al., 1991); Solanum tuberosum StEN1, AY676603; Zinnia ZEN1, AB003131 (Aoyagi et al., 1998).
No. AY986984). Furthermore, a cDNA fragment, almost identical to the \textit{DeNUC1} cDNA fragment, was obtained from ‘LPB’ carnation (GenBank Accession No. AY986985). They had 98.9% similarity in nucleotide sequence and 100% similarity in deduced amino acid sequence.

The deduced DeNUC1 protein had two His residues at positions 72 and 144, which corresponded to those necessary for RNase and DNase activities in nucleases P1 and S1 (Maekawa et al., 1991). It had four Cys residues (marked with open diamonds in Fig. 1), which corresponded to those required to form two disulfide bonds for maintaining the tertiary structure of protein in nucleases P1 and S1 (Iwamatsu et al., 1991; Maekawa et al., 1991), and \textit{Arabidopsis} (Perez-Amador et al., 2000) and \textit{Zinnia} (Aoyagi et al., 1998) nucleases. Moreover, it had several Trp, His, and Asp residues (shown by asterisks in Fig. 1), which are implicated in the binding of zinc atoms in nucleases P1 and S1 (Gite and Shankar, 1992; Gite et al., 1992; Maekawa et al., 1991; Volbeda et al., 1991), and an Asn residue (# in Fig. 1), which is glycosylated in the P1 nuclease (Maekawa et al., 1991). DeNUC1 had an amino acid sequence similar to those of other nucleases; similarities were 76.4% for \textit{Solanum} StEN1 (GenBank Accession No. AY676603), 72.1% for \textit{Arabidopsis} BFN1 (Perez-Amador et al., 2000), 70.8% for daylily SA6 (Panavas et al., 1999), 72.5% for \textit{Zinnia} ZEN1 (Aoyagi et al., 1998), and 73.5% for daffodil DAFSAG1 (Hunter et al., 2002). The phylogenetic tree was constructed from deduced amino acid sequences of nucleases of different origins by the neighbor-joining method (Fig. 2). DeNUC1 clustered with StEN1 and BFN1. Southern blot analysis of genomic DNA obtained from ‘LPB’ carnation showed two major bands after digestion with each of six restriction enzymes (Fig. 3). We preliminarily confirmed that there was no restriction site for these enzymes in the 527 bp-long DeNUC1 cDNA probe.

These findings indicated that there were two \textit{DeNUC1} copies in ‘LPB’ carnation.

The profile of flower senescence and ethylene

![Fig. 2. Phylogenetic tree of homologs of DeNUC1. The phylogenetic tree was constructed from deduced amino acid sequences by the neighbor-joining method. Accession numbers for respective genes were shown in the legend to Fig. 1, except for BEN1 from barley (Accession No. D83178). The scale indicates the base-substitution rate.](image1)

![Fig. 3. Southern blot analysis of \textit{DeNUC1} gene. Genomic DNA samples were obtained from petals of ‘LPB’ carnation.](image2)

![Fig. 4. Changes in senescence profile (A) and ethylene production (B) in ‘LPB’ carnation flowers undergoing natural senescence for 8 days. Flowers at the given time of senescence were examined for appearance and ethylene production in whole flowers. Day 0, the time of full-opening of flowers. For ethylene production data, values represent the mean±SE of 3 flowers.](image3)
production in the flowers of ‘LPB’ carnation during the 9 days of incubation are depicted in Fig. 4A and B, respectively. Petal in-rolling was observed on day 7, and petal wilting culminated on day 9. Ethylene production was detected on day 4, reached the maximum on day 6, then declined rapidly. Levels of \textit{DcNUC1} transcript in ‘LPB’ petals were monitored during senescence by northern blot analysis (Fig. 5). \textit{DcNUC1} transcript was detected on day 4 when the ethylene production in the flowers began to increase (Fig. 4B). Its level markedly increased until day 8 (Fig. 5).

Recently, concern has been raised about the method of normalizing the sample run on RNA gels for northern blot analysis of genes in senescing tissues because of massive degradation of RNA that occurs during senescence (Jones, 2004). The expression of a particular mRNA may appear to be up-regulated in a senescing tissue when it is merely being maintained at steady levels or decreasing less rapidly than the total RNA levels. In this study, also, the increase in the level of \textit{DcNUC1} transcript at the later stage of petal senescence was over-estimated by the decrease in rRNA levels. We did not investigate further the increase in the levels of \textit{DcNUC1} transcript at the end of senescence because this increase was probably related with the progress of senescence in the carnation petals. These findings were in agreement with those reported by Panavas et al. (1999), who showed that the mRNA level of \textit{SA6} nuclease gene increased during senescence in tepals of daylily flowers.

Senescence of flower petals conforms to the general definition of PCD (Rubinstein, 2000). In senescence of gypsophila petals, nuclear DNA degradation was reported to be an early regulatory event rather than a result of massive cell death in the final stage of senescence (Hoeberichts et al., 2005). Nuclear DNA degradation has been observed in senescing carnation petals (Yamada, personal communication). Because gypsophila and carnation belong to the Caryophyllidoideae subfamily in the Caryophyllaceae family, it is possible that the increase in \textit{DcNUC1} expression in carnation petals may also represent its regulation in petal senescence accompanied by massive degradation of nucleic acids in the later stages of senescence.

The expression of \textit{S1}-type nuclease genes has been reported to be associated with cell death processes, such as senescence; \textit{SA6} in daylily (Panavas et al., 1999) and \textit{BFN1} in \textit{Arabidopsis} (Perez-Amador et al., 2000). The \textit{Arabidopsis} genome database indicates the presence of five \textit{S1}-type nuclease genes, among which \textit{BFN1} is induced during leaf and stem senescence (Perez-Amador et al., 2000). The daylily \textit{SA6}, which has a similar nucleotide sequence to \textit{BFN1}, is also expressed during tepal senescence (Panavas et al., 1999). In barley, two \textit{S1}-type nucleases, \textit{BEN1} and \textit{Bnuc1} were induced in association with degradation of endosperm and salt stress-induced cell death, respectively (Brown and Ho, 1986; Muramoto et al., 1999). In \textit{Zinnia}, there are three \textit{S1}-type nuclease genes, \textit{ZEN1}, \textit{ZEN2}, and \textit{ZEN3} (Perez-Amador et al., 2000). \textit{ZEN1} participates in nuclear DNA degradation during programmed cell death, whereas \textit{ZEN2} and \textit{ZEN3} are expressed during senescence as well as during xylem formation of cultured cells (Perez-Amador et al., 2000). Our study showed that \textit{DcNUC1} is expressed in senescing carnation petals. Further study to characterize enzymatic activity is necessary to reveal whether or not \textit{DcNUC1} actually belongs to the \textit{S1}-type nuclease group.

\textbf{Literature Cited}