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Short Communication

Expression of an *AtNAP* gene homolog in senescing morning glory (*Ipomoea nil*) petals of two cultivars with a different flower life span

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SUMMARY

AtNAP, a NAC family transcription factor, has been shown to promote leaf senescence in *Arabidopsis*. We isolated an *AtNAP* homolog in morning glory (*Ipomoea nil*), designated *InNAP*, and investigated its expression during petal senescence. We used two cultivars, one showing a normal short flower life span (cv. Peking Tendan) and another a longer life span (cv. Violet). *InNAP* was highly expressed in both cultivars. Expression was high before that of the senescence marker gene *InSAG12*. *InNAP* and *InSAG12* expression was high in cv. Peking Tendan before cv. Violet. The expression of both genes was therefore temporally related to the onset of the visible senescence symptoms. An inhibitor of ethylene action (silver thiosulphate, STS) delayed petal senescence in cv. Peking Tendan but had no effect in cv. Violet. STS treatment had no clear effect on the *InNAP* expression in petals of both cultivars, suggesting that endogenous ethylene may not be necessary for its induction. These data suggest the hypothesis that *InNAP* plays a role in petal senescence, independent of the role of endogenous ethylene.

Introduction

Senescence of plant organs is a regulated developmental process resulting in cell death (Lim et al., 2007; van Doorn and Woltering, 2008). During senescence the expression of many genes is altered, for example in leaves (e.g. Buchanan-Wollaston et al., 2005) and petals (e.g. van Doorn et al., 2003; Breeze et al., 2004; Yamada et al., 2007).

In senescing *Arabidopsis* leaves 96 transcription factor (TF) genes were upregulated at least threefold (Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008). The most abundant TFs were in the NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, and MYB families. Several TFs, including NAC and WRKY, were also differentially expressed during petal senescence (Wagstaff et al., 2008), but have not been shown to function as regulators of petal senescence (van Doorn and Woltering, 2008).

Guo and Gan (2006) reported that a NAC gene, *NAC-LIKE, ACTIVATED BY AP3/PI* (*AtNAP*), was highly upregulated in senescing *Arabidopsis* leaves. *AtNAP* overexpression resulted in precocious leaf senescence, whilst the leaf senescence of loss-of-function mutants was significantly delayed. These results suggest that AtNAP is a key regulator of leaf senescence. *AtNAP* activates and represses several genes (Zhang and Gan, 2012). *AtNAP* was also upregulated during silique senescence in *Arabidopsis* and seemed to play an important role in the senescence of this organ (Kou et al., 2012). *AtNAP* was also upregulated in *Arabidopsis* petals, after flower opening (Wagstaff et al., 2008).

The life span of flowers is often terminated by petal senescence. Flowers in the genus *Ipomoea*, including *I. nil*, are an example. The flowers exhibited petal inward rolling, within one day of flower opening (Matile and Winkenbach, 1971; Kende and Baumgartner, 1974; Yamada et al., 2006). In *I. nil* we previously showed varietal differences in flower life span (Shinozaki et al., 2011). All cultivars tested responded to exogenous ethylene, but treatment with aminooxyacetic acid (AOA), an ethylene biosynthesis inhibitor, was effective only on short-lived cultivars such as Peking Tendan and not in long-lived cultivars such as Violet. This suggested that endogenous ethylene did not regulate senescence in the long-lived cultivar.

We previously isolated several genes that showed changes in expression prior to the visible senescence symptoms (Yamada et al., 2007). In this study, we isolated and characterized an *I. nil* homolog of the *AtNAP* gene in *Ipomoea* petals. We studied its expression in

Abbreviations: AOA, aminooxyacetic acid; AP3, APETALA3; NAP, NAC-LIKE,ACTIVATED BY AP3/PI; PI, PISTILLATA; PCD, programmed cell death; SAG, senescence-associated gene; STS, silver thiosulphate; TF, transcription factor; TAR, transcriptional activation region.

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petals of two cultivars (cvs. Peking Tendan and Violet), which have a different flower life span.

Materials and methods

Plant materials

Seedlings of *Ipomoea nil* (L.) Roth cvs. Violet and Peking Tendan were grown in a climate-controlled room at 24 °C, about 70% relative humidity, and cool-white fluorescent light at a photon flux density of 100 μmol m^{-2} s^{-1} for 12 h per day. We designated the onset of the light period as time 0 (t = 0 h; Yamada et al., 2007).

Cloning of InNAP

EST sequences from *I. nil* cv. TKS (BJ555720, BJ564916, BJ569812, and BJ571571) homologous to *AtNAP* (NM_105616) were collected from a BLAST search in the GenBank EST database. Total RNA was extracted from petals of both cultivars using TRIzol reagent (Invitrogen) and treatment with deoxyribonuclease I (amplification grade) (Invitrogen). Synthesis of cDNA was carried out using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). PCR was performed with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems), using *TaKaRa Ex Taq* DNA polymerase (Takara Bio). For amplification of the *InNAP* sequences of the cvs. Violet and Peking Tendan, two primers, InNAP-CF and InNAP-CR (Table S1), were designed based on the EST sequences of the cv. TKS. The PCR products were cloned into the pGEM T-Easy vector (Promega) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems).

Amino acid sequence comparison and motif analysis

Deduced amino acid sequences homologous to AtNAP (NP_564966) were collected from a BLAST search (Table S2). The accession numbers of NAC proteins listed in Fig. 1B are shown in Table S3. The NAC protein sequences were aligned using the ClustalW program version 1.83 (Chenna et al., 2003). A neighbor-joining phylogenetic tree was generated using the program MEGA version 4, and the Poisson correction algorithm (Tamura et al., 2007). The bootstrap values for nodes in the phylogenetic tree are from 1000 replications. The identities between NAP protein sequences were calculated after pairwise alignments using the EMBOSS Needle program (Rice et al., 2000).

Flower opening and visible petal senescence

Flower opening and visible petal senescence were evaluated using Flower Shape Analysis System software (Shinozaki et al., 2011). Flower buds were excised at t = −12 h, placed in microtubes containing deionized water, and held in the climate-controlled room. The corollas were photographed horizontally, using the interval-recording program of a PowerShot S3 IS digital camera (Canon). The corolla area was measured as a pixel value and the relative corolla area was calculated as the ratio to the maximum corolla area, defined as 1.00.

Treatments with STS

Flower buds were excised at t = −12 h, placed in microtubes containing 0.2 mM silver thiosulphate (STS) or deionized water, and held in the climate-controlled room.

Quantitative real-time RT-PCR

For the data of Fig. 2, total RNA samples and template cDNA were prepared according to Yamada et al. (2007). PCR was performed with a SmartCycler II System (Cepheid), using SYBR *Premix Ex Taq* (Perfect Real Time) (TaKaRa). Thermal cycling conditions were 95 °C for 10 s followed by 45 cycles of 5 s at 95 °C for denaturing, and 20 s at 60 °C for annealing and extension. For the data of Fig. 3, total RNA was extracted from petals and used in cDNA synthesis as described in the Cloning of *InNAP* section above. PCR was performed with a StepOnePlus System (Applied Biosystems) using EXPRESS SYBR GreenER qPCR Supermix Universal (Invitrogen). Thermal cycling conditions were 95 °C for 20 s followed by 40 cycles of 3 s at 95 °C for denaturing, and 30 s at 60 °C for annealing and extension. The primer pairs InNAP-F and InNAP-R, and InSAG12-F and InSAG12-R (Table S1) were designed for amplification of target genes *InNAP* (AB639146) and *InSAG12*/*In33* (AB267829). Primers Actin4-F and Actin4-R were designed for amplification of *Actin 4* (AB054978), used as an internal control. The specificity of the PCR was checked by melt curve analysis of the amplified products, using the standard method. The relative transcript level was defined as the ratio between the absolute transcript level of the target gene and that of *Actin 4* within the same sample. The mean values of biological triplicates were normalized to the maximum in each analysis.

Results

Isolation of Ipomoea homolog of AtNAP

A cDNA homologous to *AtNAP* was isolated from *I. nil* cv. Violet and named *InNAP* (GenBank AB639146). *InNAP* has an 846 bp open reading frame encoding 282 amino acids. The genomic DNA sequence of the *InNAP* coding region was sequenced (GenBank AB639148). *InNAP* comprised three exons and two introns (data not shown), similar to *AtNAP*. The deduced InNAP protein contains an NAC domain composed of five subdomains (Sub-A, B, C, D, and E in Fig. 1A), which is conserved in NAC proteins (Kikuchi et al., 2000).

Quantitative RT-PCR analysis showed that the expression of *InNAP* in *Ipomoea* leaves drastically increased by the time of visible yellowing, accompanied by an increase in the expression of *InSAG12*/*In33* (Yamada et al., 2007), an *Ipomoea* homolog of an *Arabidopsis* senescence marker gene *SAG12* (Fig. S1).

Phylogenetic analysis of 36 NAC proteins revealed that InNAP clustered with the NAP subgroup and was most similar to SlNAP, the AtNAP homolog in tomato (Fig. 1A and B) The deduced amino acid sequence of InNAP shared 67.8% identity with SlNAP, 57.1% identity with AtNAP, and 36.5% identity with OsNAP. Alignment of InNAP with AtNAP and homologous protein sequences from other plant species showed a high degree of conservation in the NAC domain, whilst the sequences of the C-terminal regions, known as transcriptional activation regions (TARs), were highly heterogeneous (Fig. 1A).

Expression of InNAP and InSAG12 in petals of cv. Violet and cv. Peking Tendan

Flower opening and petal inward rolling are shown by an increase and a decrease in corolla area, respectively (Fig. 2A). 'Relative corolla area' is the ratio of the area with the maximum area of each individual flower (Fig. 2C). We designated the onset of the light period as time 0 (t = 0 h).

Flowers of cv. Violet opened mainly from t = −2 to 1 h, although slight further unfolding was found between t = 1 h and t = 4 h. Flower inward rolling was found to commence at about t = 14 h. The inward rolling was about complete (taken to be the time to visible petal senescence) by t = 18 h. The expression of *InSAG12* was very low until t = 6 h. Considerable up-regulation was observed from t = 8 h (Fig. 2D). *InNAP* showed very low expression before flower