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Short Communication

Isolation and Expression Analysis of a Tobacco AINTEGUMENTA Ortholog (NtANTL)

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The *Arabidopsis* AINTEGUMENTA (ANT) protein is essential for proper ovule development, but functions in cell proliferation and organ growth throughout the plant. Here we report the isolation of a full-length cDNA clone from tobacco (*Nicotiana tabacum* L.) that encodes a protein with high similarity to ANT and is preferentially expressed in the pistil. In situ hybridization analysis on the tobacco ovary shows that the expression pattern of the corresponding gene is different from that of *ANT* in *Arabidopsis*.

Keywords: AINTEGUMENTA — *Nicotiana tabacum* — NtANTL.

The nucleotide sequence reported in this paper has been submitted to GenBank under the accession number AY461432.

The *Arabidopsis* AINTEGUMENTA (ANT) is a transcription factor involved in various aspects of plant development. Its main function is to promote cell proliferation in developing organ primordia (Elliott et al. 1996, Klucher et al. 1996, Krizek 1999, Mizukami and Fischer 2000). Severe *ant* knockout alleles result in smaller lateral organs (leaves and floral organs) and, most strikingly, complete absence of the ovule integuments (Elliott et al. 1996, Klucher et al. 1996, Baker et al. 1997, Schneitz et al. 1997). Furthermore, ANT activity is required for proper outgrowth of ovule primordia and for development of the marginal tissue of the pistil, i.e. septum and placenta (Elliott et al. 1996, Klucher et al. 1996, Schneitz et al. 1998, Liu et al. 2000). A function that seems to be a somewhat different from its role in cell proliferation is that ANT acts redundantly with APETALA2 to repress AGAMOUS expression in the second flower whorl and promotes petal cell identity (Krizek et al. 2000).

The functions of ANT in plant development are reflected in the distribution of its mRNA. The *ANT* transcript is first found in the developing cotyledons of the embryo (Elliott et al. 1996, Long and Barton 1998) and subsequently marks all vegetative and floral organ primordia (Elliott et al. 1996, Long and Barton 2000). The *ANT* transcript is also found in growing leaves, petals and placenta and throughout the young ovule primordia. Later during ovule development, it becomes restricted to the distal part of the funiculus and the chalaza, and is found in the developing integument primordia (Elliott et al. 1996, Klucher et al. 1996, Balasubramanian and Schneitz 2000).

The ANT protein belongs to the large AP2/ERF family of plant-specific transcription factors (Riechmann and Meyerowitz 1998). This family includes proteins with either one or two AP2 domains. ANT contains two such domains and thus belongs to the AP2 subfamily. ANT has been shown to act as a transcriptional activator, binding to a unique consensus sequence via a mode of DNA recognition that is distinct from that used by proteins containing a single AP2 domain (Nole-Wilson and Krizek 2000, Krizek 2003).

As part of a study to compare ovule development in species of the large euasterid clade, which are unitegmic (Albach et al. 2001), and *Arabidopsis*, which is bitegmic, we are searching for markers of ovule development in tobacco (*Nicotiana tabacum* L., a euasterid species). AP2 subfamily genes have been isolated from various angiosperm and gymnosperm species (Shigyo and Ito 2004). However, no true ortholog for the *ANT* gene has been described in any other dicot species. Here, we report the isolation of a tobacco *ANT* ortholog and show its expression pattern in the ovary.

A 382 bp *ANT* fragment was amplified from *Arabidopsis* inflorescence cDNA using polymerase chain reaction (PCR) primers AACTTTGGTGTTTGCTATGGAT and ATTCCCCA-TATACCCCTACAT. This fragment corresponded to a part of the coding region of the transcript at the 5′ end and excluded the region coding for the AP2 domains. The fragment was used as a probe to screen approximately 10^6 plaques of a tobacco ovary cDNA library (Rieu et al. 2003) at low stringency (hybridization at 50°C and washing to 1× SSC at 50°C). The screening resulted in the isolation of three identical cDNA clones. Two full-length clones were sequenced and the longest was named *NtANT-like* (*NtANTL*; GenBank accession number AY461432).

The *NtANTL* cDNA is 2,546 bp and contains an open reading frame of 1,932 bp. The deduced 643 amino acid protein contains two AP2 domains and shows considerable homology to the *Arabidopsis* ANT protein, both inside and outside the AP2 domain region (Fig. 1A). Phylogenetic analy-

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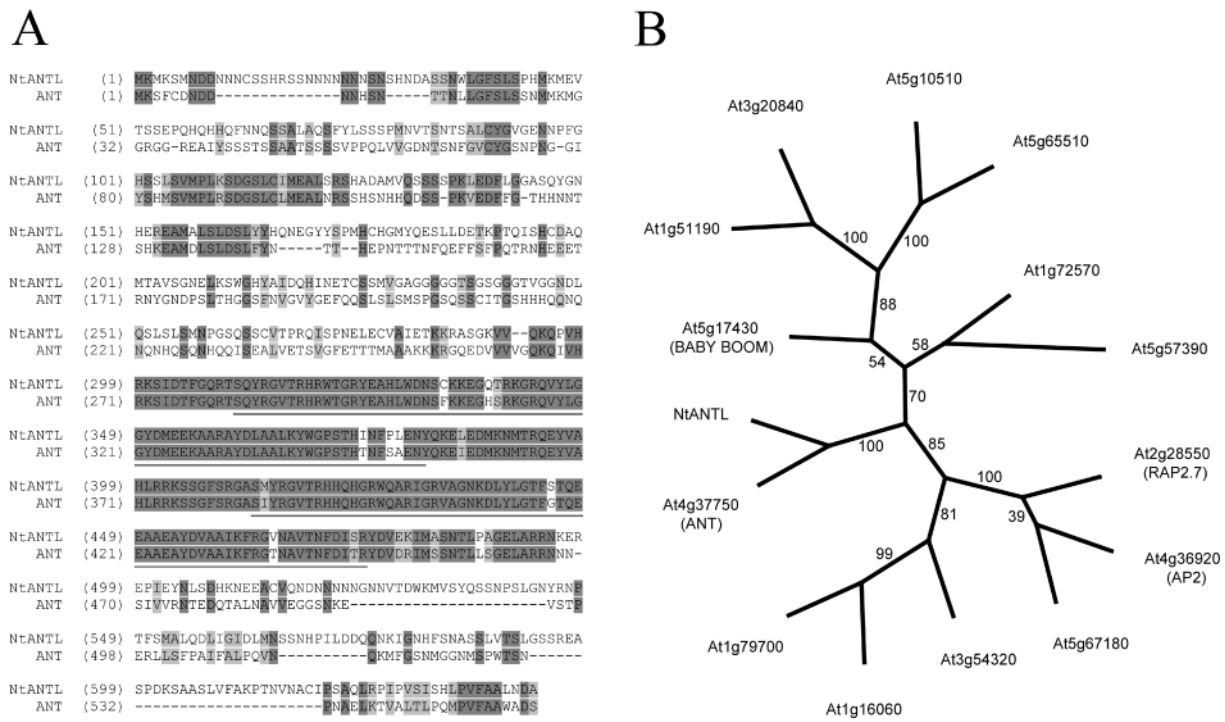

Fig. 1 (A) Alignment of deduced NtANTL and ANT proteins. The two AP2 domains (pfam00847) are underlined. Identical amino acids (43%) are shown in dark gray, conservative substitutions (9%) in light gray. (B) Phylogenetic tree of the *Arabidopsis* AP2 subfamily members and the NtANTL protein. Whole protein sequences were aligned using Clustal W (Thompson et al. 1994), and the Phylip package (version 3.6 at http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html; Felsenstein 1989) was used to calculate a distance matrix (Protdist program) and to generate a tree using the bioneighbor-joining method (Gascuel 1997). Bootstrap values are indicated as the percentage of 1,000 resamplings.

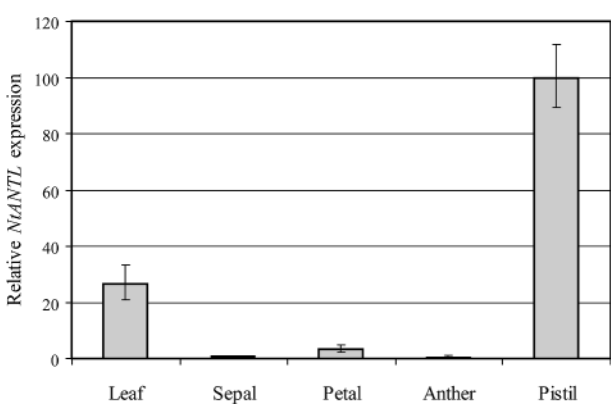

Fig. 2 Expression analysis of *NtANTL* in different tissues. Total RNA was extracted from young leaves, and from flower organs at various stages of development, excluding the earliest stages in which primordia are present. After cDNA synthesis, relative *NtANTL* transcript levels were determined using real-time quantitative PCR with SYBRgreen chemistry on an ABI 7500 Real Time PCR System (Applied Biosystems, U.S.A.) according to the manufacturer's standard protocols with four replicates of each sample. Calculations and analysis were performed as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2. The results obtained with either *NtGapC* or *NtTUBA1* as the endogenous control were virtually identical and results obtained using *NtGapC* are shown.

sis of NtANT and the 14 members of the *Arabidopsis* AP2 subfamily (Riechmann et al. 2000) shows that NtANTL clusters with the ANT protein (Fig. 1B) and, therefore, the two can be considered genic orthologs (see also Shigyo and Ito 2004).

To analyze the spatial expression pattern of *NtANTL* in tobacco, real-time quantitative reverse–transcription polymerase chain reaction (RT–PCR) was performed with primers for *NtANTL* (GGTTACCAGCCTTGGCAGTTC/GCATTGACATTTGTTGGCTTTG) and for the endogenous controls *NtGapC* (M14419; TGTGGACCTTACCGTAAGACTAGAGA/CCCTCCGATTCCTCCTTGA) and *NtTUBA1* (AJ421411; TGATCCTCGCC ATGGAAAGT/TGACATCCTTTGGCACAACATC). Fig. 2 shows that, within the flower, *NtANTL* was preferentially expressed in the ovary. Additionally, some expression was found in leaf. Because the *NtANTL* transcript was almost exclusively detected in the pistil, and the *Arabidopsis ANT* transcript has been shown to accumulate in specific regions of the ovary and ovule, a more detailed in situ hybridization analysis was performed on sections of tobacco ovaries at several stages of flower development. Fig. 3 shows that *NtANTL* transcripts were detected in all ovary tissues and seemed to be distributed evenly over the ovule tissues at all stages.

Thus, the spatial expression patterns of *NtANTL* and *ANT* are similar in that both transcripts are preferentially expressed in the ovary and hardly detectable in the other floral organs at later stages of flower development. However, expression in

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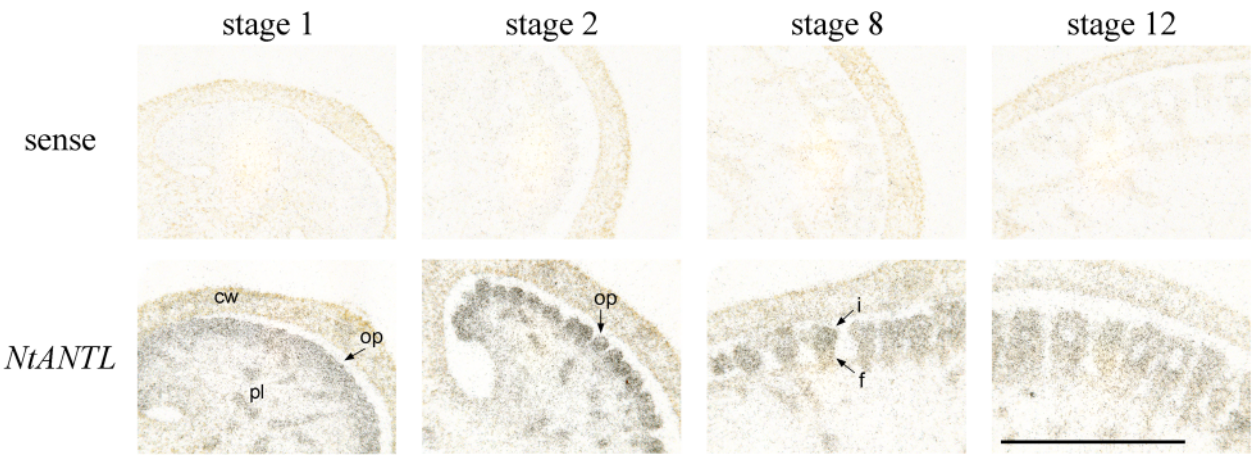

Fig. 3 In situ hybridization analysis of the *NtANTL* transcript in tobacco ovaries. Sections of ovaries at different stages of flower development (according to Koltunow et al. 1990) were hybridized with a ^{33}P-labeled sense or antisense probe, exposed for 5 d and examined using bright-field illumination as described before (Rieu et al. 2003). The hybridization signal appears as black grains. cw, carpel wall; f, funiculus; i, integument; op, ovule primordium; pl, placenta. Bar = 0.5 mm.

the tobacco ovule seems to be different from that in the *Arabidopsis* ovule: unlike *ANT*, *NtANTL* transcript accumulation is not restricted to proliferating primordia and developing integuments. However, because tobacco is an amphidiploid species, it should be noted that transcripts from two homologous parental copies of NtANTL may have been detected simultaneously in the expression analysis. Functional analysis of the *NtANTL* gene should show whether the protein is involved in ovule development and cell proliferation.

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