Conversion of Perianth into Reproductive Organs by Ectopic Expression of the Tobacco Floral Homeotic Gene \textit{NAG1}\textsuperscript{1}

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Mutations in the \textit{AGAMOUS (AG)} gene of \textit{Arabidopsis thaliana} result in the conversion of reproductive organs, stamens and carpels, into perianth organs, sepals and petals. We have isolated and characterized the putative \textit{AG} gene from \textit{Nicotiana tabacum}, \textit{NAG1}, whose deduced protein product shares 73% identical amino acid residues with the \textit{Arabidopsis AG} gene product. RNA tissue in situ hybridizations show that \textit{NAG1} RNA accumulates early in tobacco flower development in the region of the floral meristem that will later give rise to stamens and carpels. Ectopic expression of \textit{NAG1} in transgenic tobacco plants results in a conversion of sepals and petals into carpels and stamens, respectively, indicating that \textit{NAG1} is sufficient to convert perianth into reproductive floral organs.

Flowers develop from small clusters of undifferentiated cells into four organ types that occupy precisely defined positions within the flower (Bowman et al., 1989). Genetic studies have identified mutants in which the normal pattern of organ development is altered. The extensively studied homeotic mutants of \textit{Arabidopsis thaliana} have led to a simple model that describes how the products of these homeotic genes control the fate of floral organ primordia (Bowman et al., 1991). Analyses of similar mutants in \textit{Antirrhinum majus} has led to a model with many features in common to that proposed for \textit{Arabidopsis} (Coen et al., 1990; Schwarz-Sommer et al., 1990).

Wild-type tobacco (\textit{Nicotiana tabacum}) and \textit{Arabidopsis} flowers consist of four concentric whorls of organs that comprise (from outside to inside) sepals, petals, stamens, and carpels, respectively (Hicks and Sussex, 1970; Bowman et al., 1989; Koltunow et al., 1990). In \textit{Arabidopsis}, flowers of \textit{ag} mutant plants develop perianth organs (petals and sepals) in the positions normally occupied by reproductive organs (stamens and carpels). In contrast, \textit{ap2} flowers develop reproductive organs in the positions normally occupied by perianth organs. These and other genetic studies indicate that \textit{AG} and \textit{AP2} may interact in a mutually antagonistic fashion, with \textit{AP2} preventing \textit{AG} activity in whorls one and two, and \textit{AG} preventing \textit{AP2} activity in whorls three and four of wild-type flowers (Bowman et al., 1989, 1991). In addition to the role of \textit{AG} in specifying organ identity, \textit{AG} is also required for preventing the indeterminate growth of the floral meristem. Thus, whereas wild-type flowers consist of four whorls of organs, \textit{ag} mutant flowers produce an indeterminate number of whorls reiterating the pattern: (sepal, petal, petal, petal).

The \textit{AG} protein (Yanofsky et al., 1990) has significant similarity to transcription factors from yeast and animals (Dubois et al., 1987; Norman et al., 1988; Passmore et al., 1988; Jarvis et al., 1989), suggesting that \textit{AG} similarly encodes a transcription factor. The region shared by these proteins has been called the MADS domain (Schwarz-Sommer et al., 1990) and is present in a number of recently characterized plant homeotic gene products (Sommer et al., 1990; Huijser et al., 1992; Jack et al., 1992; Mandel et al., 1992a; Tröbner et al., 1992). RNA in situ hybridizations have shown that \textit{AG} is preferentially expressed in stamens and carpels (Yanofsky et al., 1990), the same organs that are lacking in \textit{ag} mutant flowers. Further studies have demonstrated that \textit{AP2} negatively regulates \textit{AG} RNA accumulation in whorls one and two of wild-type flowers (Drews et al., 1991). Here we describe the isolation and characterization of the \textit{AG} homolog from tobacco and describe the phenotype of transgenic tobacco plants that ectopically express this gene.

**MATERIALS AND METHODS**

**Screening of cDNA Libraries**

Approximately 200,000 plaques were screened with a gel-purified radiolabeled DNA probe specific for the \textit{Arabidopsis} AG cDNA (pCIT565) (Yanofsky et al., 1990). The DNA probes were labeled using the Random Primed Labeling Kit from Boehringer Mannheim according to the manufacturer’s recommendations. Hybridizations were done at 55°C in 5× SSPE, 5× Denhardt’s solution, 0.5% SDS, 25 μg/mL salmon sperm DNA for 48 h, followed by one wash in room temperature and three 55°C washes in 5× SSPE, 0.1% SDS. The \textit{Nicotiana tabacum cv Samsun} cDNA library in λCAT represents RNA isolated from immature stamens (Kelly et al., 1990; A. Kelly and R. Meeks-Wagner, personal communication).

**Cloning and Sequence Analyses**

cDNAs were subcloned into the Promega vector pGEM7Zf(+) for sequencing. Double-stranded sequencing was performed using the United States Biochemical Sequenase Version 2.0 kit according to protocol. Both strands were

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**Abbreviations:** \textit{AG}, \textit{AGAMOUS} gene; \textit{ap2}, \textit{apetala2} mutant.
sequenced for each cDNA subclone. DNA and putative protein sequences were analyzed using the MacVector program from IBI (New Haven, CT).

RNA Analyses

RNA analyses were performed on total RNA isolated from flowers and leaves from tobacco plants according to procedures previously described (Crawford et al., 1986). RNA was obtained from leaves and from immature flowers at various stages up to and including the stage at which the flowers are pollinated. Twenty micrograms of RNA were loaded per lane. RNA was size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. The gels were blotted onto Hybond-N Nylon membranes (Amersham) and hybridizations were performed as described (Crawford et al., 1986). The probe was an 800-bp HindIII fragment of NAG1 beginning at nucleotide 260 (see Fig. 1) in the pGEM7Zf(+) vector (pKY1) that did not contain the MADS-box region. RNA tissue in situ hybridizations were performed essentially as previously described (Drews et al., 1991).

Generation of Transgenic Plants

The EcoRI cDNA fragment containing the entire coding region for the NAG1 gene was inserted in the transcriptional fusion vector pMON530 (Rogers et al., 1988) in both the sense and antisense orientations. This construct was introduced into Agrobacterium strain ASE and used to transform Nicotiana tabacum var Xanthi plants by standard methods (Horsch et al., 1985). Seeds were collected from the primary regenerants, and all morphological analyses were performed in subsequent generations.

RESULTS

Sequence Analyses

The floral homeotic gene AG plays an important role in Arabidopsis flower development. As a start toward characterizing the role of this gene from a distantly related dicotyledonous plant, we isolated and characterized the putative AG homolog from tobacco (Fig. 1). A tobacco flower-specific cDNA library was screened with a probe specific for the homolog from tobacco (Fig. 1). A tobacco flower-specific cDNA library was screened with a probe specific for the homolog of the Arabidopsis AG gene (Drews et al., 1991). A tobacco flower-specific cDNA library was screened with a probe specific for the homolog of the Arabidopsis AG gene (Drews et al., 1991). A tobacco flower-specific cDNA library was screened with a probe specific for the homolog of the Arabidopsis AG gene (Drews et al., 1991).

RNA Analyses

To begin to determine the pattern of NAG1 RNA accumulation in the tobacco plant, RNA was isolated from wild-type leaves and flowers and hybridized with a NAG1-specific probe. Probes for RNA analyses were chosen from the 3' portion of the cDNA to avoid cross-hybridization with other genes containing the MADS-box. RNA blot analyses demonstrate that this gene is expressed preferentially in floral tissue, since no NAG1-specific RNA was detected in leaf tissue (data not shown). To determine the temporal and spatial pattern of NAG1 RNA accumulation in wild-type tobacco flowers, tissue in situ hybridizations were performed. NAG1 RNA is first detectable during stage 2 of flower development (Fig. 2A), at which time the first sepal primordium arises (Mandel et al., 1997). The probe was specific for the AG gene under reduced stringency conditions. The presumed translation initiation codon of the NAG1 gene corresponds to amino acid 35 of the published AG protein. NAG1 codes for a 248-amino acid deduced protein with calculated molecular mass of 28.65 kD and an isoelectric point of 9.56.

Figure 1. Sequence of the NAG1 cDNA and deduced amino acid sequence. Below the NAG1 sequence are amino acid differences in the predicted AG protein. The MADS domain is underlined. A dash (-) indicates a space inserted for aligning optimization; an asterisk (*) indicates a stop codon.
Figure 2. NAG1 expression in wild-type tobacco. In situ hybridizations were performed on 8-μm thick, longitudinal sections of N. tabacum. The tissue was subsequently stained with tolueene blue. Bright-field photos are shown on the left and dark-field photos of the same section are shown on the right. A stage-2 flower (A) has a high level of signal in the innermost cells, which will give rise to the third- and fourth-whorl organs, stamens, and carpels, respectively. A stage-5 (B) and later-stage flower (C) reveal RNA accumulation only in stamen and carpel primordia.

Ectopic Expression of NAG1

Recent studies have shown that ectopic expression of AG1 results in a conversion of sepals and petals into carpels and stamens, respectively (Mandel et al., 1992a; Mizukami and Ma, 1992). To provide further evidence that NAG1 is the cognate homolog of AG, and to further investigate the role of NAG1 in tobacco flower development, the NAG1 cDNA was transcriptionally fused to the cauliflower mosaic virus 35S promoter (Odell et al., 1985; Benfey and Chua, 1990) in

rrescence meristem. During stage 2, NAG1 RNA is detected in the center of the incipient flower in those cells that will later give rise to the stamens and carpels. By stage 5, at which time the primordia for petals and stamens are apparent, expression is uniform over the stamen primordia and in the cells that will later develop into the carpels. Later in development, NAG1 RNA accumulates in stamens and carpels and is not detected in sepals and petals (Fig. 2, B and C). Within the developing carpels, expression is strongest in the region forming the ovary.
both the sense and antisense orientations and introduced into wild-type tobacco plants. Thirty-three independently transformed plants were regenerated that express the NAG1 cDNA in the sense orientation. Although the majority of these plants displayed a strong phenotype analogous to severe alleles of the Arabidopsis ap2 mutant, a few plants displayed a less severe phenotype, similar to weak alleles of ap2.

Before describing the phenotype of the transgenic plants, it is first necessary to briefly review the structure of wild-type tobacco flowers (Fig. 3A), which has been described in detail previously (Hicks and Sussex, 1970; Kolstunow et al., 1990; Mandel et al., 1992a). The first whorl of wild-type flowers is occupied by five sepals that are connately fused for most of their lengths. The second whorl is occupied by five petals that are also connately fused along much of their lengths, and these organs alternate with the first-whorl sepals. Five stamens, each consisting of a long filament capped by a pollen-bearing anther, occupy the third whorl, and these organs are adnately fused to the second-whorl petals. The fourth whorl is occupied by a two-carpellate gynoecium.

The overall structure of the flowers with the sense orientation of the 35S-NAG1 construct consists of carpelloid first- and fourth-whorl organs (Fig. 3, B and E) and staminoid second- and third-whorl organs (Fig. 3, F and G). In most cases, the numbers and positions of organs in each whorl was the same as in wild-type flowers. However, as a result of the fusion of first-whorl carpelloid organs, the growth and development of the inner-whorl organs was impeded, causing them to be distorted in shape and to turn brown prematurely. Thus, the resulting flowers resembled enlarged gynoecia. Although the second-whorl petals are replaced by stamens, the second- and third-whorl organs remain adnately fused as in wild type. However, whereas in wild-type flowers the fused second-whorl organs completely enclose the inner whorls of organs, the second-whorl organs of the transgenic flowers consist of long filaments with anthers that are not fused along most of their lengths (Fig. 3G). In transgenic plants that displayed a less severe phenotype, the second-whorl organs consisted of staminoid petals, similar to the phenotype described for weak alleles of the Arabidopsis ap2 mutant (Bowman et al., 1991).

In contrast to Arabidopsis, flowers of tobacco are usually subtended by leaf-like organs called bracts (Fig. 3C). The bracts on 35S-NAG1 transgenic plants are abnormally shaped (Fig. 3B) and often display a conversion toward carpelloid organs with differentiated stigmatic tissue (compare Fig. 3, C and D). In contrast, vegetative leaves did not show this conversion.

In Arabidopsis, mutations in the AG gene result in petals developing in place of stamens and a new flower developing in place of the gynoecium. To determine if a decrease in NAG1 expression in tobacco results in a similar phenotype, flowers of 35S-NAG1 antisense transgenic plants were analyzed. Forty-six transgenic lines carrying the 35S-NAG1 antisense construct were generated, although only 10 of these displayed a phenotype that differed from wild-type flowers. Plants carrying the 35S-NAG1 antisense construct showed a partial conversion of stamens into petals (Fig. 3, H–K), as anticipated for a partial suppression of the wild-type NAG1 gene activity. The extent of this phenotype varied considerably from a nearly complete conversion of stamens into petals to a partial conversion in which the third-whorl organs consisted of a nearly wild-type appearing stamen with petaloid tissue at the tips of the anthers. Although the fourth-whorl organs developed as carpels as in the wild type, the resulting carpels often did not fuse properly (Fig. 3I). It is unclear why the carpels were not converted into new flowers, as in the Arabidopsis ag mutant, but perhaps the levels of expression of the antisense transgene are insufficient to inactivate endogenous NAG1 activity.

**DISCUSSION**

The Arabidopsis floral homeotic gene AG encodes a putative transcription factor that has several roles in flower development. First, AG is involved in the specification of stamens and carpels, since these organs fail to develop in ag mutant flowers. Second, AG suppresses the indeterminate growth of the floral meristem in that ag mutants display an indeterminate number of whorls resulting in a "flower within a flower" phenotype. Third, genetic studies indicate that AG prevents the activity of AP2 in the third and fourth whorls of wild-type flowers (Bowman et al., 1991).

Similar classes of homeotic mutants have been described in a wide variety of taxonomically distant plant species, suggesting that homologous mechanisms exist to control flower development (Meyerowitz et al., 1989; Coen and Meyerowitz, 1991). Indeed, the AG, APETALAL3, LEAFY, and APETALAL1 genes of Arabidopsis all have counterparts in the distantly related plant A. majus (Coen et al., 1990; Sommer et al., 1990; Huijser et al., 1992; Jack et al., 1992; Mandel et al., 1992b; Weigel et al., 1992; Bradley et al., 1993).

To provide additional insights into the nature of AG gene function, and to determine if the observations in other plant species can be extended to another dicotyledonous plant, we isolated and characterized the AG gene from N. tabacinum, designated NAG1. The deduced NAG1 protein shares 73% identical amino acid residues with its Arabidopsis counterpart, and within the 56-amino acid MADS domain they are 100% identical. Since this region is part of a sequence-specific DNA binding domain, this suggests that NAG1 and AG recognize the same nucleotide sequence.

Like AG, the NAG1 gene is preferentially expressed in flowers. NAG1 RNA is first detected in young flowers at about the time during which the first sepal primordium arises. At this time, NAG1 RNA is detected only in the center of the incipient flower in those cells that later will give rise to stamens and carpels. At all later stages of flower development, NAG1 RNA accumulates only in stamens and carpels and is never detected in sepals and petals. Thus, the expression pattern of NAG1 RNA closely resembles that of AG in Arabidopsis (Yanofsky et al., 1990; Drews et al., 1991) and the recently isolated PLENA gene from Antirrhinum, the cognate homolog of AG (Bradley et al., 1993).

Transgenic plants that ectopically express the NAG1 gene from the cauliflower mosaic virus 35S promoter display homeotic conversions of petals into reproductive organs. Similar alterations in flower development were observed in transgenic plants that ectopically express either the Arabidopsis or Brassica AG genes (Mandel et al., 1992a; Mizukami and
Ma, 1992). It has also been recently shown that dominant mutations in the Antirrhinum gene PLENA result in a similar conversion of perianth into reproductive organs (Bradley et al., 1993). Furthermore, plants that express the NAG1 cDNA in the antisense orientation show a partial conversion of stamens into petals, a phenotype resembling that of mutations in the AG gene of Arabidopsis. Taken together, the nucleotide sequence analysis, RNA expression data, and
transgenic phenotypes all support the conclusion that NAG1 represents the tobacco homolog of the Arabidopsis AG gene. Furthermore, since AG homologs in distantly related plant species are expressed in a similar manner, the regulatory interactions leading to the temporal and spatial pattern of AG expression have apparently been conserved throughout the evolution of these distantly related plant species. 

The absence of all three classes of organ identity genes, such as in the ap2 ap3 ag triple mutant, produces a ‘ground state’ floral organ (a carpelloid leaf) (Bowman et al., 1991; Coen and Meyerowitz, 1991). Although the plants transgenic for the 35S-NAG1 construct do not convert vegetative leaves into carpelloid organs, the bracts are converted into carpelloid organs. This suggests that the previously described ground state floral organ may resemble a bract rather than a vegetative leaf. In addition, these data suggest that one or more factors present in bracts but absent from vegetative leaves are required together with NAG1 to convert vegetative leaves into carpels.

Genetic studies in a wide variety of distantly related dicotyledonous plants have identified homeotic mutants that display similar alterations in floral structure. We have shown that tobacco has a homolog of the Arabidopsis floral homeotic gene AG, and that the tobacco NAG1 gene functions to specify the identity of reproductive organs. These studies lend further support to the conclusions that the factors controlling flower development have been highly conserved throughout the evolution of dicotyledonous plants.

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LITERATURE CITED


