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ORIGINAL ARTICLE

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Constitutive expression of the *SAP1* gene from willow (*Salix discolor*) causes early flowering in *Arabidopsis thaliana*

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Abstract *SAP1-1* and *SAP1-2* were isolated from the male reproductive buds of willow (*Salix discolor*, clone S365). *SAP1-1* differs from *SAP1-2* based on a few nucleotide substitutions, but the sizes of their full-length cDNAs are identical. The deduced amino acid sequences of *SAP1-1* and *SAP1-2* were 98% similar and contain the same C-terminal amino acid motif "GYGA" like that of *PTAP1-2* from *Populus trichocarpa*. The expression patterns of *SAP1* in various parts of the male reproductive buds of *S. discolor* implicate this gene in the formation of the inflorescence meristems, bracts, and floral meristems. To characterize the functions of *SAP1*, we assessed *Arabidopsis thaliana* transformed with 35S::*SAP1-1*. A total of 52 transgenic T_1 lines were obtained, and a 3:1 segregation ratio was obtained in the T_2 generation of each line. In the T_3 generation, five homozygous transgenic lines were obtained, which were used for further analysis. Screening of transgenic lines was greatly facilitated by the detection of GFP expression starting with germinating seeds. Phenotypes of the homozygous transgenic lines included early flowering, conversion of inflorescence branches to solitary flowers, formation of terminal flowers, and formation of flowers with greater number of petals, stamens, and pistils. Northern analysis showed similar expression levels in all five lines. This study provides the first functional analysis of an *APETALA1* (*AP1*)/*SQUAMOSA* (*SQUA*) homolog from a dioecious species and suggests that *SAP1* is a homolog of the *AP1*/*SQUA* gene.

Keywords *AP1* · Dioecious · Early flowering · *Salix discolor* · *SAP1*

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Introduction

It has been demonstrated that *LEAFY* (*LFY*) and *APETALA1* (*AP1*) play central roles in the initiation and development of flowers (Mandel et al. 1992; Weigel et al. 1992). In *lfy ap1* double mutants, inflorescence shoots develop in place of flowers, and the phenotype is more severe than in either single mutant (Huala and Sussex 1992; Weigel et al. 1992). In *Arabidopsis thaliana*, constitutive expression of either *LFY* or *AP1* is sufficient to confer floral meristem identity to the shoot meristems (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Therefore, both genes are involved in the transition from vegetative to reproductive development. It appears that *LFY* and *AP1* do not only have overlapping functions, but they also reinforce each other's activities (Cho et al. 1999). *AP1* acts in the floral meristem identity pathway as a direct downstream target of *LFY* (Wagner et al. 1999). Specifically, *LFY* is able to bind to the *cis*-regulatory elements that control *AP1* expression (Parcy et al. 1998). The *ap1-1* mutation partly suppresses the floral phenotype of 35S::*LFY* transgenic *Arabidopsis* (Weigel and Nilsson 1995), suggesting that *AP1* also regulates the activity of *LFY*. On the other hand, the activity of *AP1* is not restricted to the early steps of determining the identity of floral meristems like that of *LFY*. *AP1* is also involved in later stages of floral development through specifying the identities of sepals and petals (Bowman et al. 1993).

Unlike *LFY*, *AP1* belongs to the MADS-box gene family of transcription factors that regulate different aspects of flower development (Riechmann and Meyerowitz 1997). *SQUAMOSA* (*SQUA*) is a homolog of *AP1* in *Antirrhinum majus*, which is involved in the same activity as *AP1* (Huijser et al. 1992). The *AP1*/*SQUA* belongs to the class A gene in the ABC model of floral development (Gustafson-Brown et al. 1994; Weigel and Meyerowitz 1994). In spite of the similarities between *AP1* and *SQUA*, there appears to be a difference in their ability to regulate flowering. *AP1* is involved in establishing the identities of floral meristems and organs in *A. thaliana*. Loss-of-function *ap1* mutations caused a disruption of sepal and petal development

(Bowman et al. 1993). In *A. majus*, *SQUA* is not absolutely essential for flower development (Huijser et al. 1992).

Willows are dioecious plants, which means that each individual produces only male or female flowers. There are no sepals and petals in willow flowers. Our previous study on male reproductive development in willow described the formation of two stamens per flower with no indication of the pistil being initiated and aborted (Zhang and Fernando 2005). There is no published information available on the *AP1*/*SQUA* homolog in willow or other dioecious plants. Molecular and genetic analysis of the *AP1*/*SQUA* homolog from willow will provide insights to understand the regulation of flower development in dioecious woody plants that lack sepals and petals.

In willows, the mechanism of floral development and patterns of gene expression during the initiation of the inflorescence and flowers is unknown. Information on this will contribute to our understanding of the mechanism behind flower development in dioecious plants. This study aims to (1) isolate *SAP1*, the homolog of the *AP1*/*SQUA* gene from *Salix discolor* (clone S365); (2) analyze the sequence and structure of *SAP1*; (3) characterize the expression patterns of *SAP1* in *S. discolor*; and (4) determine the functions of *SAP1* as expressed in *A. thaliana*.

Materials and methods

Plant materials and growth conditions

Vegetative and male reproductive buds of willow (*S. discolor*, clone S365) at various stages of development were collected from late July to early August in 2003 and 2004 from SUNY-ESF's Tully Genetics Field Station in Tully, NY. The buds were used for DNA and RNA extraction. Young leaves were collected in August 2004 for genomic DNA extraction. The buds used for in situ hybridization analysis were fixed in freshly prepared 4% (w/v) paraformaldehyde in 1× phosphate-buffered saline buffer (pH 7). *A. thaliana* wild type (ecotype Columbia) and transformed *A. thaliana* were grown in a controlled temperature room at 22°C with 16 h of light for long-day (LD) or 8 h of light for short-day (SD) conditions. The plants grown under SD condition were exposed to LD condition at the first sign of flower initiation.

Isolation and sequencing of *SAP1*

Total RNA was isolated from male reproductive buds bearing inflorescence meristems and developing flowers following an improved method by Salzman et al. (1999). A total of 0.3 g of reproductive buds (bracts removed) was ground into a fine powder using liquid nitrogen. First-strand cDNA was synthesized through reverse transcription-PCR using Clontech SMART PCR cDNA Synthesis Kit (Clontech, San Jose, CA). Two degenerate primers (MADS9 and MADS123) specific to the MADS-box and K-box, respectively, were designed based on the conserved sequences of these domains from several *AP1*/*SQUA* homologs (Table 1). A 366-bp PCR product was obtained and cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). DNA sequencing was done at the BioResource Center, Cornell University, Ithaca, NY. The DNA sequence was analyzed through BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Rapid amplification of cDNA ends (RACE) technique was used to obtain the complete sequence of the open reading frame of *SAP1*. Two specific primers, MADSGSP1 and MADSGSP2 (Table 1), were designed based on the partial sequence of *SAP1*. 5′-RACE and 3′-RACE PCR products were obtained using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). The PCR products were sequenced and analyzed as above. The full-length cDNA was obtained by PCR with end-to-end primers (5SAP1 and 3SAP1) (Table 1) using first-strand cDNA as the template. The PCR product was cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen). A total of ten single colonies were picked and sequenced to confirm the sequences. Two different full-length cDNA sequences were obtained and analyzed through BLAST. For intron analysis, an 873-bp DNA fragment was obtained by PCR with primers 5SAP1 and 3SAP1 (Table 1) using genomic DNA as the template. This amplified product was sequenced as above.

Sequence and phylogenetic analysis

The complete coding sequences of *SAP1-1* and *SAP1-2* were individually aligned with nucleotide sequences of homologs from various plants through BLAST. Multiple alignments based on deduced amino acid sequences were done through UNIPROT (http://www.pir.uniprot.org/search/blast.shtml). SAP1-1, SAP1-2, and 11 other AP1/SQUA homolog proteins from various woody and herbaceous flowering plants were chosen for comparison. A phylogenetic tree was constructed based on the 13 AP1/SQUA homolog proteins using PAUP (version 4.0) according to Swofford (1998). Parsimony analysis and a branch-and-bound search were used to build the best tree, and bootstrap analysis was performed to estimate the resampling of estimated log-likelihood (Hasegawa and Kishino 1994; Himi et al. 2001).

A. thaliana transformation

A construct (pCGSAP1) was made to accomplish constitutive expression of SAP1-1 in *A. thaliana*. GFP expression was used as the basis to screen for putative transformants. The primers 5SAP3 and 3SAP3 (Table 1) were used to amplify the open reading frame of *SAP1-1*. To confirm the orientation and sequence of *SAP1-1* in the pCGSAP1 construct, we used the primers 5SAP3 and TER1 for PCR and sequencing. The plasmid DNAs were introduced into *Agrobacterium tumefaciens* EHA105 by CELL-Porator Electroporation System (GibcoBRL, Gaithersburg, MD).

Table 1 Oligonucleotide sequences used to isolate and characterize *SAP1-1* and *SAP1-2* from *Salix discolor*

Primer name	Oligonucleotide sequence
MADS9	5′-AARMGIATHGARAAYAARATHAAYMG-3′
MADS123	5′-TGITGYTCIARIBWYTGIARITCYTT-3′
MADSGSP1	5′-TATACTCCAGGGTCCAGTTCCCCGGTGA-3′
MADSGSP2	5′-ACTCAGTCCTCTGTGATGCTGAGGTTGC-3′
5SAP1	5′-GAGATGGGAAGAGGTAGGGTTCAG-3′
3SAP1	5′-GGACAGCGTAATTCATCATCATGT-3′
5SAP3	5′-TAACTCGAGGAGATGGGAAGAGGTAGG-3′
3SAP3	5′-TAACTCGAGGGACAGCGTAATTCATCA-3′
TER1	5′-ACTCACACATTATTATGGAGAAACTCGAG-3′
RNSAP4	5′-GGGGGAGGATCTAGACTCCATGAG-3′
RNSAP5	5′-CTCAAGCTTCATGCTCCATAGCCT-3′

I=inosine, B=C or G or T, H=A or C or T, M=A or C, R=A or G, W=A or T, Y=C or T

A. tumefaciens-mediated transformation of *A. thaliana* was performed essentially according to Clough and Bent (1998) and Desfeux et al. (2000). T_1 seeds were placed on moist filter paper and screened under a fluorescence microscope (Leica DMLB, San Jose, CA) based on GFP expression. GFP expressing seeds were transferred to pots with potting mix and grown in a controlled temperature room. T_1, T_2, and T_3 seeds were screened and segregation analysis was obtained based on GFP expression. The phenotypes under LD and SD conditions were observed from T_3 homozygous *A. thaliana* transgenic lines, wild type, and transgenic control line. Images representing the various phenotypes were obtained through a DEI-7500 CE digital video camera (Optronics, Goleta, CA).

In situ hybridization

The primers RNSAP4 and RNSAP5 (Table 1) were used to amplify a 421-bp *SAP1-1* cDNA fragment with *Xba*I and *Hin*dIII restriction sites. The PCR product was cloned into pSPT18 and pSPT19, and RNA labeling was performed through in vitro transcription using the DIG RNA Labeling Kit (Roche, Indianapolis, IN). T7 RNA polymerase was used to obtain the antisense RNA probe and SP6 for RNA sense probe. The reproductive buds, which were fixed in 4% paraformaldehyde (pH 7) for 14 h, were dehydrated through a graded series of ethanol, infiltrated, and embedded in paraffin. RNA in situ hybridizations with non-radioactive probes were performed as described by Jackson (1991). The sections were examined under a light microscope (Leica DMLB), and representative images were obtained using a DEI-7500 CE digital video camera (Optronics, Goleta, CA).

Northern analysis

Total RNA was isolated from five *SAP1 A. thaliana* transgenic lines and a transgenic control line using RNeasy Plant Mini Kit (Qiagen, Valencia, CA). A total of 20 μg of total RNA for each sample was loaded on a 1.2% formaldehyde agarose gel. Total RNA was blotted on positively charged nylon membranes (Roche). The 421-bp PCR product amplified from *SAP1-1* cDNA was used as a probe for Northern analysis and labeled with digoxigenin-11-UTP using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche). Hybridization and immunological detection were performed according to the manufacturer. The membrane was scanned using a GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA). All digital photographic images were cropped and assembled using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA).

Results

Isolation and sequence analysis of *SAP1-1* and *SAP1-2*

SAP1-1 and *SAP1-2* from *S. discolor* were isolated and sequenced (GenBank accession numbers DQ068268 and DQ068269, respectively). *SAP1-1* is different from *SAP1-2* based on a few nucleotide positions, but their full-length cDNAs are both 750 bp long.

The deduced amino acid sequences of *SAP1-1* and *SAP1-2* cDNAs were compared with each other and with 11 other AP1/SQUA homolog proteins from woody and herbaceous flowering plants using UNIPROT (Fig. 1). Comparison of sequences showed that SAP1-1 and SAP1-2 were 98% identical, but there were 100% identities in the MADS-box and K-box sequences between SAP1-1 and SAP1-2. On the other hand, at the nucleotide level, there are four differences in the K-box region between *SAP1-1* and *SAP1-2* (Fig. 1). The MADS-box was located from amino acid 1 to 60, and K-box was located from amino acid 91 to 155 (Fig. 1). The difference in the sequences of the deduced amino acid sequences of *SAP1-1* and *SAP1-2* occurred in the I and C domains. SAP1-1 and SAP1-2 have high identity with various AP1/SQUA homolog proteins (Table 2).

To determine the evolutionary relationships among the AP1/SQUA homolog proteins, we constructed a phylogenetic tree based on the deduced amino acid sequences (Fig. 2). The tree showed that the SAP1-1 and SAP1-2 proteins were most closely related since they were in the same clade. Both of these proteins were very closely related to PTAP1-2 and followed by PTAP1-1. All of the AP1/

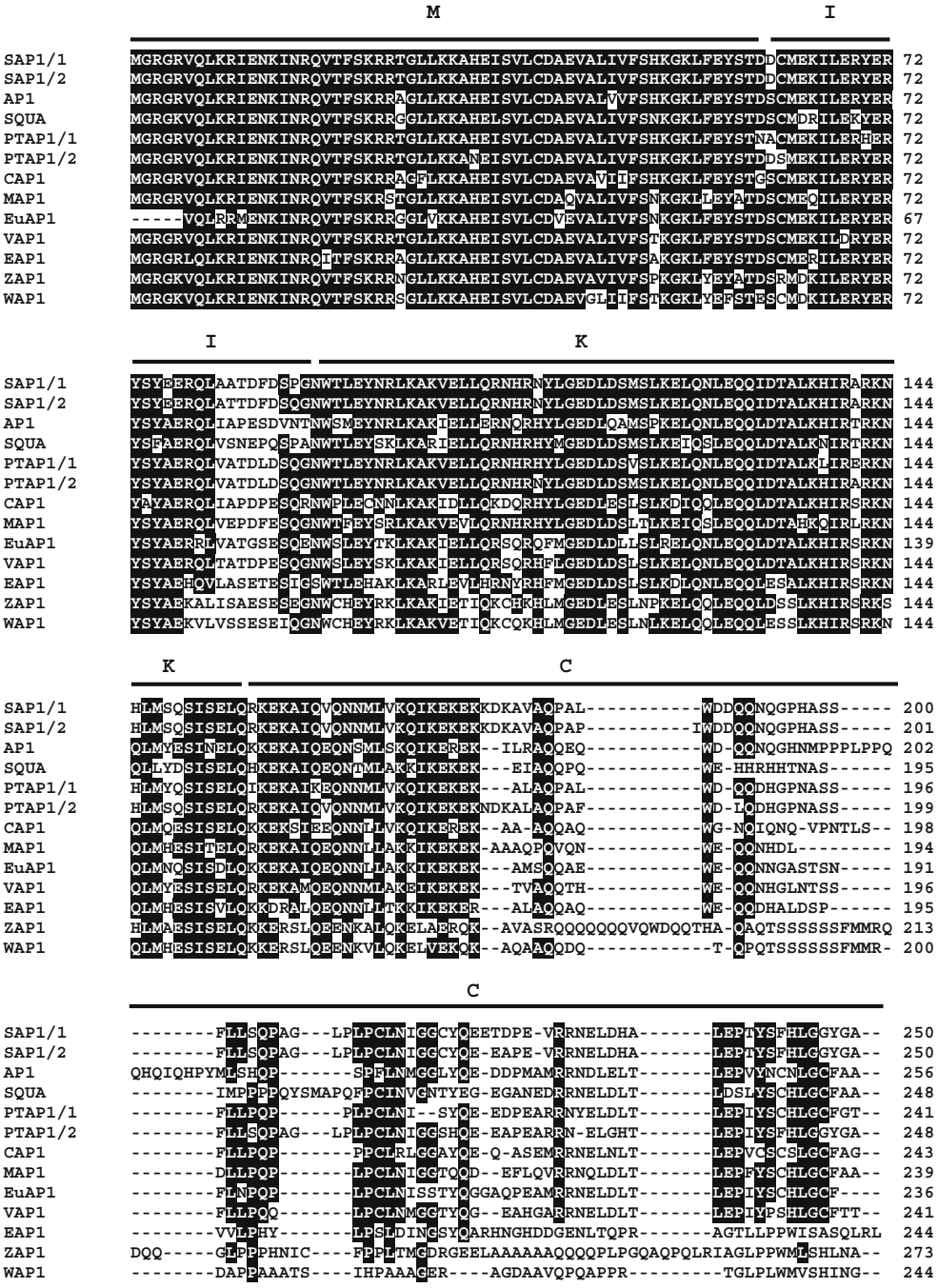

Fig. 1 Alignment of the deduced amino acid sequences of AP1, SQUA, and their homologs including SAP1-1 and SAP1-2 (accession numbers in parentheses): AP1 from *A. thaliana* (Z16421, Mandel et al. 1992), SQUA from *Antirrhinum majus* (X63701, Huijser et al. 1992), PTAP1-1 from *P. trichocarpa* (AY615964), PTAP1-2 from *P. trichocarpa* (AY615966), CAP1 from *Citrus sinensis* (AY338975), MAP1 from *Malus domestica* (AY071921, Sung et al. 1999), EuAP1 from *Heuchera americana* (AY306148, Litt and Irish 2003), VAP1 from *V. vinifera* (AY538746, Calonje et al. 2004), EAP1 from *Eucalyptus globulus* (AF305076, Kyozuka et al. 1997), ZAP1 from *Z. mays* (L46400, Mena et al. 1995), and WAP1 from *T. aestivum* (AB007504, Murai et al. 2003). *Black shadows* indicate identical amino acids; *dashed lines* indicate gaps to optimize the alignment. *M* MADS-box, *I* I region, *K* K-box, *C* C-terminus

SQUA homolog proteins from eudicot species, including *SAP1-1* and *SAP1-2*, were in the same clade. It is logical that ZAP1 and WAP1 from the monocots *Zea mays* and *Triticum aestivum*, respectively, were in another clade in the phylogenetic tree.

Expression patterns of *SAP1* in *S. discolor*

The expression patterns of *SAP1* were determined by examining the male reproductive buds of *S. discolor*. *SAP1* RNA was detected by using a nonradioactive antisense probe from inflorescence meristem representing various developmental stages. Strong *SAP1* expression was localized in all the layers of the inflorescence meristem (Fig. 3a), as well as in the developing flowers along the flanks of the inflorescence meristem (Fig. 3b). *SAP1* was also strongly detected in bract primordia (Fig. 3b), young bracts (Fig. 3c), and floral meristems (Fig. 3c). Using the sense probe, no positive hybridization was observed in any part of the male reproductive buds that were examined (Fig. 3d).

Table 2 Percent identity of deduced amino acid sequence among SAP1-1, SAP1-2, and various AP1/SQUA homologs

Protein name	Species	Accession number	Identity with SAP1-1 (%)	Identity with SAP1-2 (%)
PTAP1-2	*Populus trichocarpa*	AY615966	91	91
PTAP1-1	*P. trichocarpa*	AY615944	84	83
VAP1	*Vitis vinifera*	AY538746	75	76
MAP1	*Malus domestica*	AY071921	71	72
CAP1	*Citrus sinensis*	AY338975	70	70
EuAP1	*Heuchera americana*	AY306148	70	70
AP1	*Arabidopsis thaliana*	Z16421	68	68
EAP1	*Eucalyptus globulus*	AF305076	67	66
SQUA	*Antirrhinum majus*	X63701	64	65
WAP1	*Triticum aestivum*	AB007504	61	60
ZAP1	*Zea mays*	L46400	60	60

Expression of *SAP1-1* in *A. thaliana*

A construct (pCGSAP1) for the overexpression of *SAP1-1* driven by the cauliflower mosaic virus (CaMV) 35S promoter was designed. The T-DNA also contains *mgfp5-er* driven by the CaMV 35S promoter. Another construct, pCGFP, contained *mgfp5-er* driven by CaMV 35S promoter as a negative control for plant transformation (data not shown).

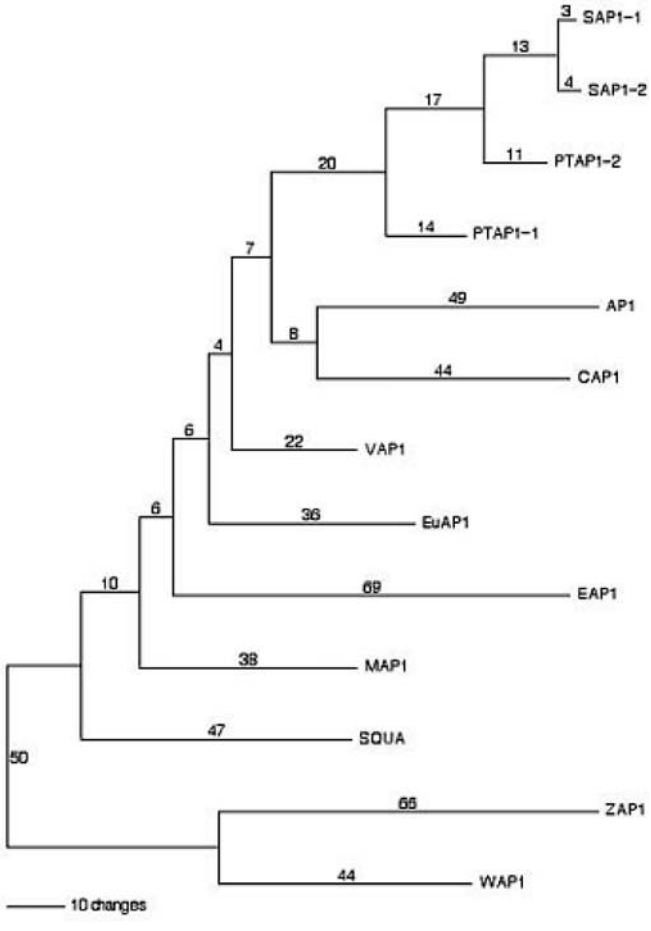

Fig. 2 Phylogenetic relationships among AP1/SQUA homolog proteins. Branch length values are indicated

To assess whether *SAP1-1* can induce inflorescence and floral development similar to the effects of *AP1*/*SQUA*, we generated transgenic *A. thaliana* plants in which *SAP1-1* was expressed under the control of the CaMV 35S promoter. GFP expression was used to screen the transformants. Besides the wild type as a control, an *A. thaliana* transgenic control line in which T-DNA contained 35 S::*GFP* was also produced. A total of 52 independent transgenic lines were obtained based on screening for GFP expression. The GFP expressing T_1 seeds were grown under LD condition to generate T_2 and T_3 plants. A 3:1 segregation ratio of GFP fluorescence was obtained in each line in the T_2 generation (Table 3). The segregation analysis was based on GFP expression through examination of several hundreds of seeds. A total of nine T_2 *A. thaliana* transgenic lines were chosen based on the floral phenotypes and GFP expression. In the T_3 generation, five homozygous transgenic lines were obtained, and these were used for further analysis. For each of these lines, 40 seeds were sown and grown in the greenhouse under LD and SD conditions. Seeds, leaves, flowers, and siliques were further screened for GFP expression (data not shown). Phenotypes of the homozygous transgenic lines included early flowering, conversion of inflorescence branches to solitary flowers, formation of terminal flowers, and formation of flowers with greater number of sepals, petals, stamens, and pistils.

The 35S::*SAP1-1A. thaliana* transgenic lines dramatically triggered early flowering in all the five *A. thaliana* transgenic lines. There was no difference observed in the timing of flowering and floral phenotypes between wild type and transgenic control line (Table 4). In fact, flowering occurred while there were only two rosette leaves formed, and this was the case both under LD and SD conditions. The differences between the transgenic lines were primarily in the phenotypes of the flowers. In line 35S:: *SAP1-1*.1, terminal flowers showed a typical phenotype (Fig. 4a), but solitary flowers were formed in this line (Fig. 4b). Wild type at the same age was still in the vegetative stage (Fig. 4c). In line 35S::*SAP1-1*.2, terminal flowers formed but with six petals, while all the other floral organs had typical numbers (Fig. 4d). Solitary flowers were formed, and they had a typical number of floral organs. In line 35S::*SAP1-1*.3,

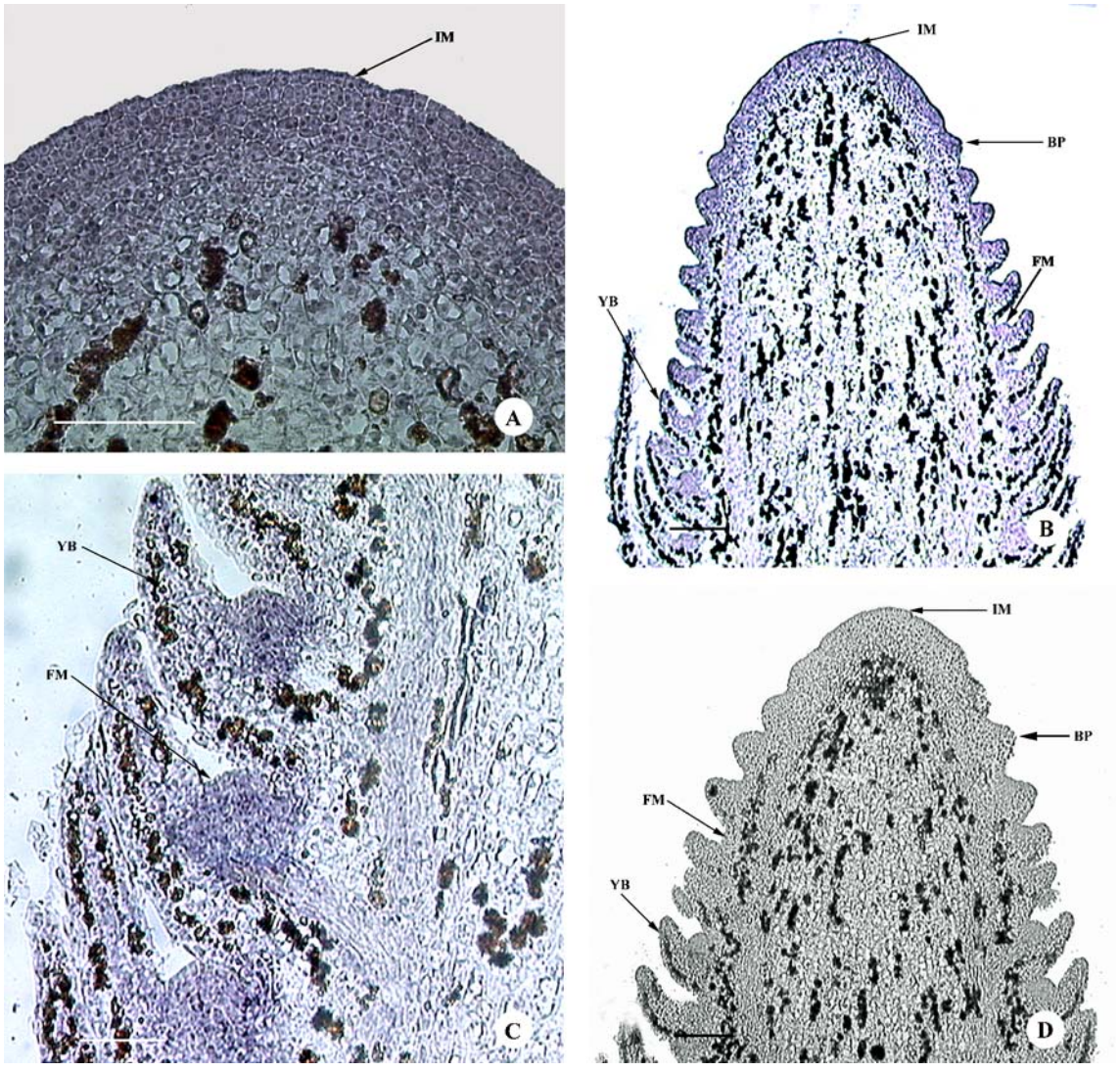

Fig. 3 In situ hybridization analysis of *SAP1* expression in male reproductive buds of *S. discolor*. All images are longitudinal sections. **a** Magnified inflorescence meristem, antisense probe. **b** Early male inflorescence, antisense probe. **c** Young bracts and floral meristems, antisense probe. **d** Early male inflorescence, sense probe. *BP* bract primordial, *FM* floral meristem, *IM* inflorescence meristem, *YB* young bract. *Bars*=100 μm

terminal flowers formed three pistils, with more petals and stamens as compared with wild type (Fig. 4e). From the base of the main inflorescence stalk, curled bracts subtend the solitary flowers with typical floral phenotypes (Fig. 4f). In line 35S::*SAP1-1*.4, terminal flowers formed four sepals, eight petals, 12 stamens, and two pistils (Fig. 4g). Like in other lines, conversion of inflorescence branches to solitary flowers also occurred in line 35S::*SAP1-1*.5 (Fig. 4h). Secondary flowers formed from the inner whorl of the main terminal flower in this line (Fig. 4i). Under SD

Table 3 Segregation ratios of GFP expression in the T_2 generation of five 35S::*SAP1-1* transgenic lines

Transgenic line	GFP-positive	GFP-negative	Segregation ratio	95% CI	*p* value*
35S::*SAP1-1*.1	389	129	3.02:1	0.711, 0.788	0.960
35S::*SAP1-1*.2	352	118	2.98:1	0.707, 0.788	1.000
35S::*SAP1-1*.3	411	136	3.02:1	0.713, 0.787	0.961
35S::*SAP1-1*.4	377	124	3.04:1	0.712, 0.790	0.918
35S::*SAP1-1*.5	364	121	3.01:1	0.710, 0.788	1.000

The test is for the proportion of positive GFP expression that is equal to 0.75 vs not equal, and the results in all transgenic lines are not significant (*p* value >0.888), which indicate that the data are consistent with the hypothesized 3:1 segregation ratio
*Test of p=0.75 vs $p\neq$0.75

Table 4 Phenotypes of various T_3 homozygous 35S::*SAP1-1* transgenic lines of *A. thaliana* and controls in long-day growing conditions

	Number	Days to flowering	Rosette leaves at flowering	Inflorescence branches after 6 weeks	Solitary flowers	Terminal flowers
Genotypes						
35S::*SAP1-1*.1	38	8.3±0.6**	2.0±0.0**	1	+	+
35S::*SAP1-1*.2	38	8.5±1.2**	2.0±0.0**	1	+	+
35S::*SAP1-1*.3	39	8.4±1.1**	2.0±0.0**	1	+	+
35S::*SAP1-1*.4	36	8.7±1.5**	2.0±0.0**	1	+	+
35S::*SAP1-1*.5	40	8.1±0.4**	2.0±0.0**	1	+	+
Transgenic control	36	29.6±1.1	14.4±0.7	3.3±0.9	−	−
Wild type	40	28.7±1.3	13.9±1.2	3.2±0.7	−	−

**Significance at 1% level (p value <0.01) compared with the controls

condition, all the transgenic lines showed phenotypes similar to those observed under LD conditions, although initiation of flowering occurred 4–5 days later.

To determine the *SAP1* expression level in the five T_3 35S::*SAP1-1A. thaliana* transgenic lines, we performed Northern analysis using a digoxigenin-11-UTP-labeled probe. The results showed that *SAP1* was expressed at

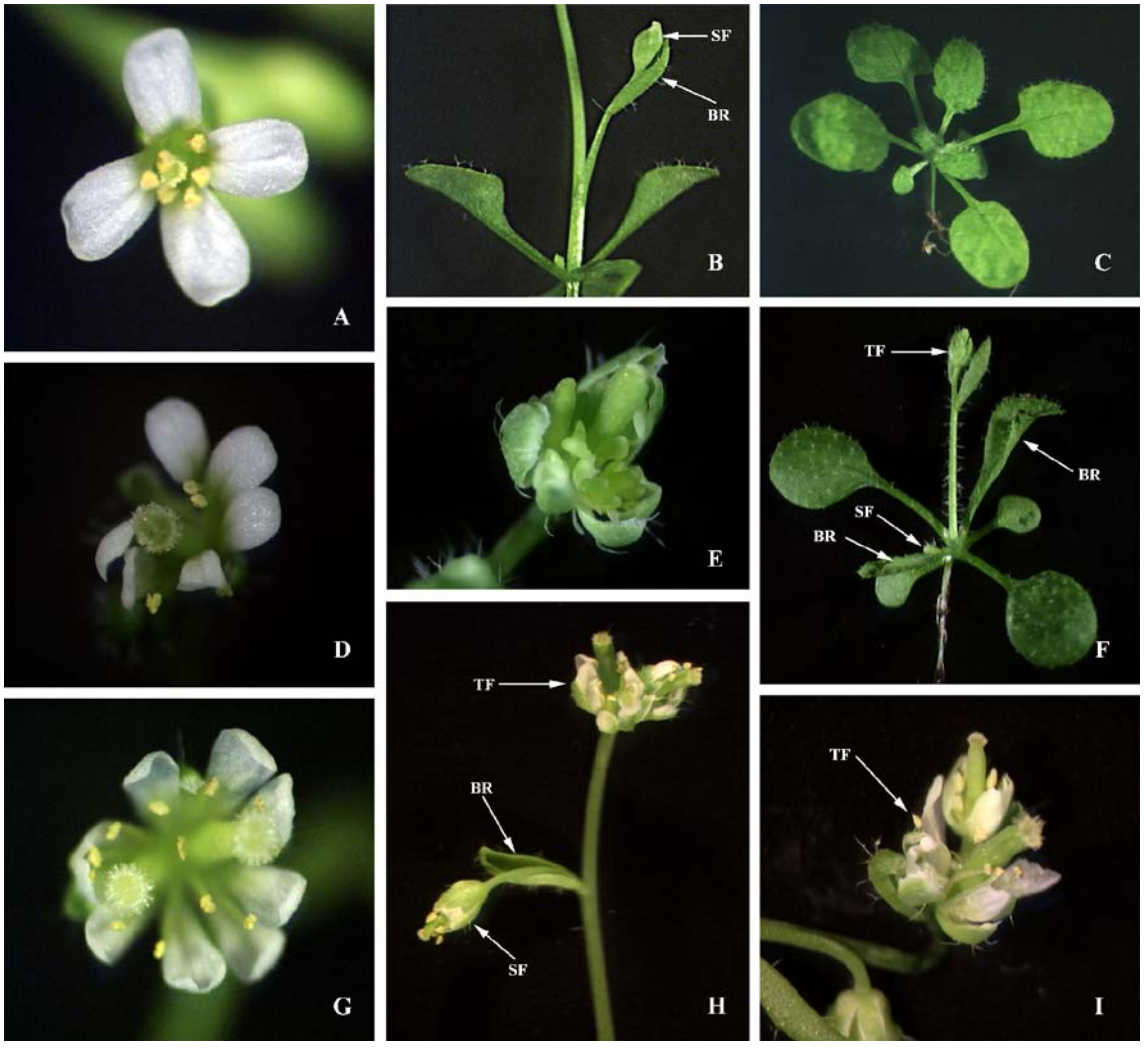

Fig. 4 Heterologous expression of *SAP1-1* in *A. thaliana* transgenic lines. **a** Line 35S::*SAP1-1*.1 terminal flower with typical phenotype. **b** Line 35S::*SAP1-1*.1 with conversion of inflorescence branch to solitary flower. **c** Wild type of the same age as transgenic lines. **d** Line 35S::*SAP1-1*.2 terminal flower with six petals. **e** Line 35S::*SAP1-1*.3 terminal flower with three pistils, more petals and stamens. **f** Line 35S::*SAP1-1*.3 curled bracts subtending the solitary flowers. **g** Line 35S::*SAP1-1*.4 terminal flower with four sepals, eight petals, 12 stamens, and two pistils. **h** Line 35S::*SAP1-1*.5 with the formation of terminal flowers and a converted inflorescence branch to solitary flower. **i** Line 35S::*SAP1-1*.5 with the secondary flower from inner whorl of the main terminal flower. *BR* bract, *TF* terminal flower, *SD* secondary flower, *SF* solitary flower

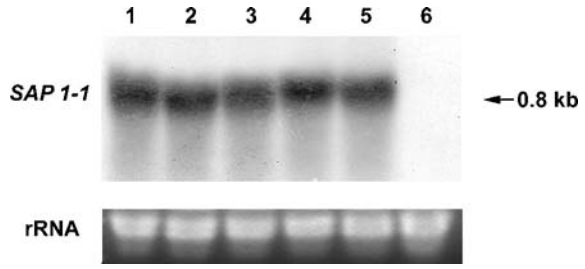

Fig. 5 Northern analysis of five T_3 35S::*SAP1-1 A. thaliana* transgenic lines. *Lane 1* 35S::*SAP1-1*.1, *lane 2* 35S::*SAP1-1*.2, *lane 3* 35S::*SAP1-1*.3, *lane 4* 35S::*SAP1-1*.4, *lane 5* 35S::*SAP1-1.5*, *lane 6* transgenic control line. A total of 20 μg of total RNA was loaded per lane. A nonradioactive probe was used. NBT/BCIP color substrate solution was used for detection. The lower panel displays the rRNA of each sample to indicate equal loading volumes

similar levels in the five transgenic lines (Fig. 5). Segregation analysis of GFP expression in the T_2 generation showed a 3:1 ratio in the five transgenic lines.

Discussion

SAP1 paralogs and phylogenetic relationships

The two paralogs of *SAP1* from *S. discolor* belong to the MADS-box family of transcription factors. The deduced amino acid sequences of *SAP1-1* and *SAP1-2* share 98% identity. The difference occurs in the sequences of the I and C-terminus regions. The MADS-box is responsible for DNA binding, dimerization, and accessory factor-binding function (Shore and Sharrocks 1995). The K-box seems to be plant-specific (Theissen et al. 1996) and is also involved in protein dimerization (Ma et al. 1991; Shore and Sharrocks 1995). The I region is less conserved, and the C-terminus is the most variable which is involved in ternary complex formation and transcriptional activation (Egea-Cortines et al. 1999). However, neither SAP1-1 nor SAP1-2 have the prenylation motif "CFAA" at the C-terminus, which are found in AP1 and SQUA (Rodríguez-Concepción et al. 1999; Yalovsky et al. 2000). On the other hand, it appears that the absence of the prenylation motif is common to many AP1/SQUA homologs. This motif plays an important role in the determination of the function and specificity of *AP1* in *A. thaliana* (Yalovsky et al. 2000). Instead of the prenylation motif "CFAA" found in AP1 and a few other AP1/SQUA homologs, SAP1-1 and SAP1-2 have a C-terminal amino acid motif "GYGA." This motif is also found in PTAP1-2 from *P. trichocarpa*. It appears that the C-terminal amino acid motif "GYGA" is, so far, found only in *Salix* and *Populus*, which are dioecious with flowers devoid of sepals and petals. ZAP1 from *Z. mays* and WAP1 from *T. aestivum* share a similar amino acid sequence "HLNA(G)" instead of "CFAA." This variation is congruent with the report that the C-terminus may have played an important role in the functional diversification of the MADS-box genes (Davies and Schwarz-Sommer 1994). The studies on *AP1*/*SQUA* homologs such as *PEAM4* from *Pisum sativum* (Berbel et al. 2001) and *NtMADS11* from *Nicotiana tabacum* (Jang et al. 2002) suggest that prenylation is not an essential factor in the function of *AP1*, but rather that it may reinforce *AP1* activity.

The phylogenetic tree shows the relationships of the *AP1*/*SQUA* homologs from several eudicots and monocots. In the phylogenetic tree, the SAP1-1 and SAP1-2 proteins are in the same clade, and both are most closely related to PTAP1-1 and PTAP1-2 from *P. trichocarpa*. This demonstrates a very high degree of sequence conservation between willows and poplars. Our result supports the placement of willow and poplar in the same family. Also, the homologs from eudicots are well separated from those of the monocots.

SAP1 expression patterns in *S. discolor*

The localization of *SAP1* in various parts of the male reproductive buds of willow implicates this gene in the formation of the inflorescence meristems, bracts, and floral meristems. These vary from the expression patterns of *AP1* in *A. thaliana*, i.e., it is normally expressed in young flower primordia but not in the inflorescence meristems (Mandel et al. 1992). However, the expression patterns of some *AP1*/*SQUA* homologs such as *CDM111* from *Dendrathema grandiflorum*, *BpMADS3* from *Betula pendula*, and *VAP1* from *Vitis vinifera* demonstrate that RNA from these *AP1*/*SQUA* homologs can also be detected in the inflorescence meristems (Sung et al. 1999; Elo et al. 2001; Calonje et al. 2004; Shchennikova et al. 2004). In herbaceous plants, *CDM111* is expressed in the inflorescence meristems and developing bracts (Shchennikova et al. 2004). In woody species, *BpMADS3* is expressed in both male and female inflorescences (Elo et al. 2001). *VAP1* is also expressed in the inflorescence meristems (Calonje et al. 2004). Such an expression pattern has also been observed in other woody perennials like apple (Sung et al. 1999). Therefore, *SAP1* expression patterns in the inflorescence meristems and floral meristems are similar to other *AP1*/*SQUA* homologs, especially from woody species.

In *A. thaliana*, *AP1* RNA accumulates in sepals and petals throughout their development (Mandel et al. 1992). However, sepals and petals are absent in *Salix* flowers as well as in *Populus*. Although the sequences of *PTAP1-1* and *PTAP1-2* have been submitted to GenBank in May 2004, no published report is available describing their expression patterns in *P. trichocarpa*.

SAP1-1 expression in *A. thaliana*

The functions of *AP1*/*SQUA* homologs from several species have been characterized through heterologous expression using *N. tabacum* (Sung et al. 1999), *A. thaliana* (Fornara et al. 2004), or both (Berbel et al. 2001; Hsu et al. 2003). By constitutively expressing *SAP1-1* in *A. thaliana*, it was assessed whether this willow homolog gene can stimulate flowering, as has been shown for *AP1* (Mandel and Yanofsky 1995). In *A. thaliana*, overexpressing *AP1* dramatically causes early flowering and conversion of in-

florescence meristems to flower meristems (Mandel and Yanofsky 1995). Expression of *SAP1-1* in *A. thaliana* was analyzed from five homozygous T_3 transgenic lines. The phenotypes of these transgenic lines showed early flowering that was about 20 days earlier than the controls. This suggests that *SAP1-1* is functional and is involved in the transition from vegetative to reproductive development. Similar phenotypes were also observed in *A. thaliana* transformed with *PEAM4*, an *AP1*/*SQUA* homolog from *P. sativum* (Berbel et al. 2001). Whether or not the early flowering phenotype is a direct consequence of *AP1*/*SQUA* acting at the vegetative shoot apex or is an indirect result caused by interaction with other genes is still not clear. However, it is a useful tool for reducing the time to flowering of either woody or herbaceous plants.

In addition to early flowering, the 35S::*SAP1-1 A. thaliana* transgenic lines produce flowers of varying phenotypes. Conversion of inflorescence branches to solitary flowers and formation of terminal flowers are also produced by the transgenic lines. The same phenotypes were also observed in several *AP1*/*SQUA* homologs that were overexpressed in *A. thaliana* (Berbel et al. 2001; Elo et al. 2001; Hsu et al. 2003). For example, constitutive expression of *BpMADS3* in *A. thaliana* resulted in the formation of a terminal flower (Elo et al. 2001). Constitutive expression of *PEAM4* caused the formation of terminal flowers and the appearance of solitary flowers in *A. thaliana* (Berbel et al. 2001). These phenotypes are similar to those described in *A. thaliana* overexpressing *AP1* (Mandel and Yanofsky 1995). Our results demonstrate that *SAP1-1* is involved in various aspects of flower development in transgenic *A. thaliana*, suggesting that *SAP1-1* is functional as a floral meristem identity gene.

Surprisingly, more than four petals per flower were observed in several 35S::*SAP1-1 A. thaliana* transgenic lines. These phenotypes were not found in *A. thaliana* transformed with *AP1* or other *AP1*/*SQUA* homologs. The ability of 35S::*SAP1-1* to induce the formation of more petals in *A. thaliana* demonstrates that it also functions in controlling the number of petals. This is interesting since petals, as well as sepals, are not formed in willow. Therefore, the absence of sepals and petals in willow flowers is not due to the silenced *SAP1-1* expression but may be due to the lack of other gene products that interact with *SAP1-1*. One possible gene product is *SEP3*, which has been shown to be required for petal development (Pelaz et al. 2001).

More stamens also occurred in several transformed *A. thaliana*. It is possible that *SAP1-1* is able to regulate the expression of other MADS-box genes in *A. thaliana*, such as *AP3* and *PI*, which are involved in the development of stamens. It is well established that *AP1* activates the expression of floral homeotic genes such as *AP3* and *PI* (Irish and Sussex 1990; Bowman et al. 1993; Krizek and Meyerowitz 1996; Ng and Yanofsky 2001).

Some 35S::*SAP1-1 A. thaliana* transgenic lines produced not only more petals but also more pistils. It is possible that *SAP1-1* is able to interact with *SEP3* in transgenic *A. thaliana*. It has been shown that *SEP3* is also required for pistil development (Pelaz et al. 2001). However, *SAP1-1* does not regulate the formation of pistils in male individuals probably because the *AG*, as well as other gene products such as *SEP3*, may not be expressed in willow. Further characterization of floral gene expression would resolve these questions in willow. This current study provides the first functional analysis of an *AP1*/*SQUA* homolog from a dioecious species.

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