

RESEARCH PAPER

# Interaction study of MADS-domain proteins in tomato

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## Abstract

MADS-domain proteins are important transcription factors involved in many biological processes of plants. Interactions between MADS-domain proteins are essential for their functions. In tomato (*Solanum lycopersicum*), the number of MIKC<sup>c</sup>-type MADS-domain proteins identified has totalled 36, but a large-scale interaction assay is lacking. In this study, 22 tomato MADS-domain proteins were selected from six functionally important subfamilies of the MADS-box gene family, to create the first large-scale tomato protein interaction network. Compared with *Arabidopsis* and *petunia* (*Petunia hybrida*), protein interaction patterns in tomato displayed both conservation and divergence. The majority of proteins that can be identified as putative orthologues exhibited conserved interaction patterns, and modifications were mostly found in genes underlining traits unique to tomato. *JOINTLESS* and *RIN*, characterized for their roles in abscission zone development and fruit ripening, respectively, showed enlarged interaction networks in comparison with their *Arabidopsis* and *petunia* counterparts. Novel interactions were also found for members of the expanded subfamilies, such as those represented by AP1/FUL and AP3/PI MADS-domain proteins. In search for higher order complexes, TM5 was found to be the preferred bridge among the five SEP-like proteins. Additionally, 16 proteins with the MADS-domain removed were used to assess the role of the MADS-domain in protein–protein interactions. The current work provides important knowledge for further functional and evolutionary study of the MADS-box genes in tomato.

Key words: Flower development, higher order complexes, MADS-domain proteins, protein–protein interaction, tomato, yeast two-hybrid.

## Introduction

The MADS-box gene transcription factor family is one of the most widely studied gene families in plants (Parenicova *et al.*, 2003; Kaufmann *et al.*, 2005; de Folter *et al.*, 2006). MADS-box genes play fundamental roles in a number of biological processes from root development to fruit ripening. Higher plant MADS-box genes are considered to be derived from two lineages: the type I and type II MADS-box genes (Alvarez-Buylla *et al.*, 2000). The type II or MIKC MADS-box genes have been extensively studied and functionally characterized. Based on their intron–exon structures, MIKC-type genes can be further classified into MIKC<sup>c</sup>-type and MIKC<sup>\*</sup>-type (Henschel *et al.*, 2002). The divergence of flower structures in higher plants should be attributed significantly to the radiation of the MIKC<sup>c</sup>-type MADS-box genes (Becker and Theissen, 2003). MIKC<sup>c</sup>-type genes are those frequently reported to play important roles in plant development, whereas the MIKC<sup>\*</sup>-type genes were first discovered in mosses and clubmosses, and their functions remain unknown (Henschel *et al.*, 2002). The MIKC<sup>c</sup>-type proteins have undergone extensive duplications, followed by functional diversifications that led to a complicated working network (Becker and Theissen, 2003). The completely sequenced *Arabidopsis*, rice, and poplar genomes were reported to have 39, 47, and 64 MIKC<sup>c</sup>-type MADS-box genes, respectively (Kofuji *et al.*, 2003; Parenicova *et al.*, 2003; Leseberg *et al.*, 2006). To perform their functions, MADS-domain proteins are

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known to form complexes, preferably with other MADS-domain proteins. The 'Floral Quartet Model' is widely accepted, in which members of the SEPALLATA (SEP) subfamily act as bridges for the formation of tetramers of MADS-domain proteins (Honma and Goto, 2001; Theissen and Saedler, 2001; Kaufmann *et al.*, 2005). Studies in recent years have revealed an increasing number of possible arrangements of MADS-domain proteins as hetero/homodimers or higher order complexes (Favaro *et al.*, 2002; Shchennikova *et al.*, 2004; de Folter *et al.*, 2006).

The MIKC type of MADS-domain proteins were so named for their MADS-domain (M), intervening (I), keratin-like (K), and highly variable C-terminal domains (Theissen *et al.*, 1996). The MADS-domain, with its putative DNA-binding function, is the most conserved feature of all MADS-domain proteins across the kingdoms. Interactions between MADS-domain proteins are largely achieved via the K domain. For example, interactions between *Arabidopsis* MADS-domain protein AGAMOUS (AG) and four other proteins, AGL2 (SEP1), AGL4 (SEP2), AGL6, and AGL9 (SEP3), were first detected by the yeast two-hybrid (Y2H) approach using constructs without the MADS-domain (Fan *et al.*, 1997). These interactions were confirmed to be authentic in later studies using full-length proteins (de Folter *et al.*, 2005). Previous work indicated that floral organ identity determination by MADS-domain proteins may be independent of their DNA-binding specificity (Krizek and Meyerowitz, 1996; Riechmann *et al.*, 1996). Additional studies have indicated that although the K domain is sufficient for protein–protein interaction, the presence of the MADS-domain may affect interaction patterns. For example, additional interactions of PISTILLATA (PI) and APETALA3 (AP3) with SEP proteins were observed using MADS-deleted constructs in *Arabidopsis* when compared with full-length constructs (Yang *et al.*, 2003; Yang and Jack, 2004; de Folter *et al.*, 2005). A large-scale *Arabidopsis* assay using the GAL4 system demonstrated that full-length constructs offered reproducible interaction patterns, some of which conflicted with previous results using truncated constructs (de Folter *et al.*, 2005). Within the past several years, Y2H assays have been performed using constructs either with or without the MADS-domain, with no clear justification for the chosen methodology.

Tomato MADS-box genes were among the earliest to be studied. As a matter of fact, some unique MADS-box lineages were first discovered in tomato, such as *TM6* and *TM3* (Pnueli *et al.*, 1991, 1994). Using degenerate primers, Hileman *et al.* (2006) were able to amplify 36 tomato MADS-box genes successfully. Large-scale protein interaction analysis can help provide insights into functions and evolution of MADS-box genes. Studies on MADS-domain protein interactions have been carried out

systematically in *Arabidopsis* (de Folter *et al.*, 2005) and extensively in petunia (Immink *et al.*, 2003), another member of the Solanaceae. Such knowledge in tomato is very limited, with only a selected group of MADS-domain proteins (Busi *et al.*, 2003; de Martino *et al.*, 2006). Here the protein–protein interaction network of 22 tomato MIKC<sup>c</sup>-type MADS-domain proteins from various subfamilies is reported. The results showed conserved interaction patterns among functionally essential MADS-domain proteins in tomato. Distinguishable divergence of direct and ternary interactions among the lineage-specific MADS-domain proteins was observed. A comparison of the interaction patterns of putative orthologous MADS-domain proteins from tomato, petunia, and *Arabidopsis* is presented. In addition, 16 proteins with their MADS-domain removed were used to assess the effect of the MADS-domain in protein interaction specificity.

## Materials and methods

### Tomato MADS-box gene sequences

Most MADS-box genes used in this study were either previously published or selected from Solanaceae Genomic Network (SGN) unigenes based on their sequence similarity to known flower MADS-domain proteins (see Supplementary Fig. S1 at *JXB* online). During the course of the study, Hileman *et al.* (2006) identified and named some of the unigenes which had not been characterized previously and thus their naming system (SLMBP#) was used here. In the case of published genes, unigenes were used to confirm the validity of the sequence. Two genes *TM5* (X60480) and *TM4* (X60757) that were reported by Pnueli *et al.* (1991) were both found to be inconsistent with expressed sequence tag (EST) data, which was confirmed by Busi *et al.* (2003). The previously published *TM3* sequence indicated a poly(A) tract in the K domain that resulted in a string of lysines. To determine the C-terminal sequence of *TM3*, a reverse primer (StTM3R2 5'-GGGGTTCTCTCTGTTGAAGACCACC-3') was designed according to a potato (*Solanum tuberosum*) unigene SGN-U272194 that most closely resembled the published tomato *TM3*. This primer amplified an extended *TM3* sequence that provided a better protein for Y2H assays.

### Phylogenetic analysis

Twenty-two tomato and related petunia and *Arabidopsis* MADS-domain proteins were aligned using Clustal W (Thompson *et al.*, 1994, 1997) and manually edited using Jalview (Clamp *et al.*, 2004) to include the first ~170 amino acid sequences (MIK domains). Neighbor–Joining (NJ; Saitou and Nei, 1987) trees were generated using the MEGA3.1 program (Kumar *et al.*, 2004) with p-distance and the complete deletion option. Bootstrap analyses (Felsenstein, 1985) were performed with 1000 bootstrap replicates to assess support values. The *Arabidopsis* type I MADS-domain protein PHERES was used as an outgroup.

### Development of bait and prey constructs

Total RNA was isolated from root, stem, leaf, floral meristem, and mature flowers of tomato (*Solanum lycopersicum*) cv. LA3021 using RNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA). Poly(A) RNA was converted to cDNA using the Reverse

Transcription System (Promega, Madison, WI, USA). Most forward primers contained an *EcoRI* site and reverse primers contained *Sall* restriction sites. Twenty-two selected genes were amplified using standard PCR conditions. Digestion products were purified and ligated into the *EcoRI/Sall*-cut pBD-Gal4Cam and pAD-Gal4-2.1 (Stratagene, La Jolla, CA, USA). Exceptions were SLMBP3, TM29, LePI, TPI, and SLMBP21 which contained an *EcoRI* site in the region to be cloned, and thus primers were designed with *Sall* sites on the 5' and 3' ends. This insert was cloned into *Sall*-digested pBD and pAD. Ligation products were transformed into XL-1 Blue *Escherichia coli* (Stratagene) and transformants were screened using PCR. Transformants were sequenced to confirm that they were cloned in-frame and void of any mutations. Bait and prey constructs were transformed into PJ69-4A(MATa) and PJ69-4A(MAT $\alpha$ ; James *et al.*, 1996) by means of LiAc transformation (Clontech, Mountain View, CA, USA). Bait and prey transformants were positively selected on SD medium lacking Trp and Leu, respectively. Bait proteins were tested for self-activation by detecting growth on SD Trp<sup>-</sup>His<sup>-</sup> with 10 mM 3-amino-1,2,4-triazole (3-AT) and Trp<sup>-</sup>Ade<sup>-</sup> directly. Bait constructs were also tested by mating them with empty AD constructs and plating on Trp<sup>-</sup>Leu<sup>-</sup>His<sup>-</sup> and Trp<sup>-</sup>Leu<sup>-</sup>His<sup>-</sup>Ade<sup>-</sup> with various amounts of 3-AT. TM3 (MIKC1/2 and IKC1/2) and TM5 (MIKCA225) both showed low levels of autoactivation at lower levels of 3-AT ( $\leq 3$  mM); therefore, positives were only scored for higher levels of 3-AT and/or using the *ADE* reporter gene.

The various constructs used and their lengths are listed in Supplementary Fig. S1 at *JXB* online. In some cases, the C-terminus was truncated in an attempt to eliminate the possibility for autoactivation. Thus, constructs with C-terminal truncations should not necessarily be considered proteins with native activation domains. For truncated C-terminus constructs, the program PSIPRED [Jones, 1999] <http://bioinf.cs.ucl.ac.uk/psipred/> was used to predict the secondary structure of the MADS-domain proteins. The codon position where the  $\alpha$ -helix ends, which extends from the K to the C-terminus, was noted and primers were designed to capture as much of the helix region as possible. The resulting constructs usually had ~20 residues of the C-terminus.

#### Yeast two-hybrid and three-hybrid assays

Two-hybrid (Y2H) assays were carried out using yeast (*Saccharomyces cerevisiae*) strain PJ69-4A (James *et al.*, 1996). Yeast cells containing the bait and prey vectors were grown overnight (30 °C, 250 rpm). Mating was conducted by dropping 7  $\mu$ l of each culture serially onto solid YPD (Clontech). Cells were grown at 30 °C overnight and transferred to SD medium lacking Trp and Leu to select for diploids containing prey and bait vectors. After 2 d, fresh diploid cells were plated on SD medium lacking Trp, Leu, and His, with 0, 1, or 3 mM 3-AT, and SD medium lacking Trp, Leu, His, and Ade (see Supplementary Table S1 at *JXB* online). Plates were incubated for up to 10 d at room temperature (23 °C) and 90% of detected interactions activated both the *HIS* and *ADE* reporter genes. An empty prey and bait vector were used as negative controls with each bait and prey construct, respectively. Positives were scored for an interaction when clear growth was detected in the absence of any growth for the negative control. The assays were repeated at least three times with fresh transformants.

Yeast three-hybrid (Y3H) screens were carried out for prey constructs shown to be negative in the Y2H assays. Initial screens were performed systematically by transforming yeast cells containing a bait construct with a pTFT1 vector (Egea-Cortines *et al.*, 1999) harbouring a selected MADS-box gene. Genes were cloned into pTFT1 with the same digested gene inserts used for the pBD and pAD construct development. The exception was for the MIKC assays with TM5. The BD:TM5 MIKCA225 showed minimal

autoactivation; however, TFT1:TM5 MIKCA225 could activate reporter genes in the absence of an AD clone with 3-AT levels exceeding 50 mM in some cases. Therefore, TFT1:TM5 MIKCA208 was designed for those BD constructs found to interact with TM5 in yeast two-hybrid assays. The yeast was then mated with the prey constructs. Yeast containing BD/TFT combinations were grown overnight in SD medium lacking Trp and Ade, and the prey grown in SD medium lacking Leu. Cells were mated, in a similar manner as stated above for Y2H, with yeast allowed to grow overnight at 30 °C. Cells were then transferred to SD medium lacking Trp, Leu, and Ade, and allowed to grow for 2 d at 30 °C. Y3H interactions were assayed on SD medium lacking Trp, Leu, Ade, and His with various amounts of 3-AT at room temperature for up to 10 d.

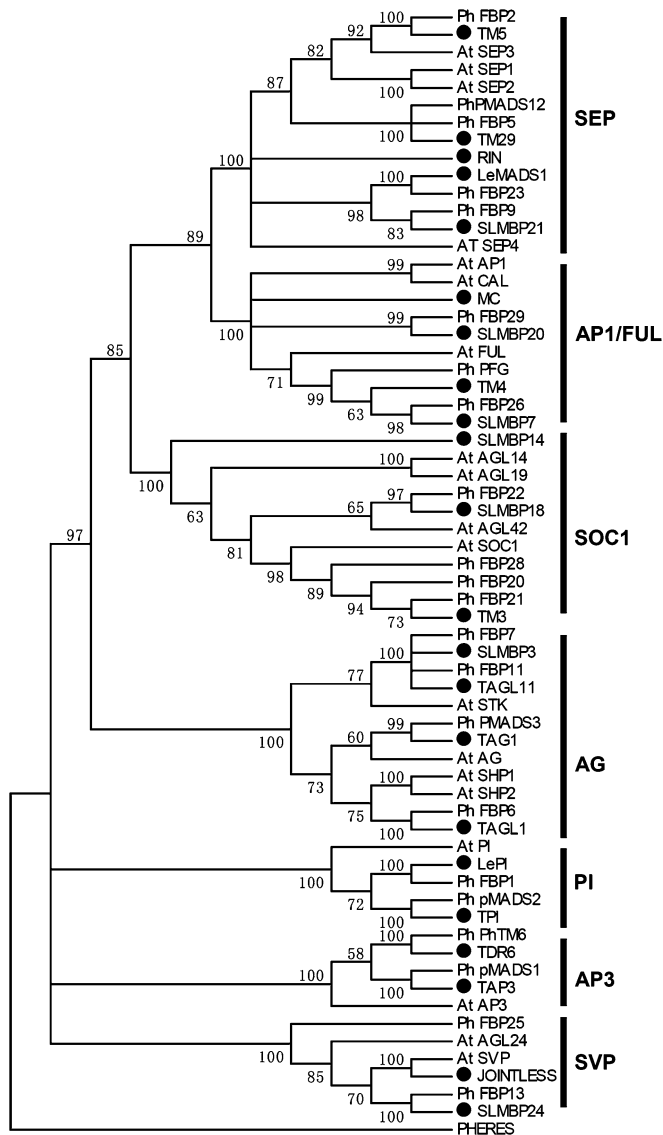
After identification of potential Y3H positives, yeast containing the bait and prey constructs were mated, and fresh cultures were grown in SD medium lacking Trp and Leu. The respective pTFT1 construct was then re-introduced into yeast cells and re-screened for a positive interaction. In all cases, each pBD/pTFT1 combination was mated with an empty pAD and each pBD/pAD combination was transformed with an empty pTFT1 as negative controls. Supplementary Fig. S2 at *JXB* online contains sample data from both the Y2H and Y3H assays.

## Results

### Tomato MADS-domain proteins selected for protein-protein interaction assay

Twenty-two MIKC<sup>c</sup>-type tomato MADS-domain proteins from various functional categories were selected according to their phylogenetic relationships with *Arabidopsis* and petunia proteins (Fig. 1; see Supplementary Fig. S1 at *JXB* online). For simplicity, phylogenetic subfamilies are referenced by representative *Arabidopsis* protein names. As shown in Fig. 1, three proteins, TM3, SLMBP18, and SLMBP14, belong to the SOC1 subfamily, represented by *Arabidopsis* SUPPRESSOR OF CONSTANS1 (SOC1), a key protein for multiple flowering time control pathways (Lee *et al.*, 2000; Samach *et al.*, 2000). TM3 has been previously identified but not functionally characterized in tomato (Pnueli *et al.*, 1991). The remaining two, SLMBP18 and SLMBP14, were identified recently and have homology to AGL42/AGL71/AGL72 and AGL13/AGL14 members of the SOC1 subfamily, respectively (Hileman *et al.*, 2006). Similar to *Arabidopsis*, there were two *SHORT VEGETATIVE STAGE* (SVP)-like genes [*JOINTLESS* (*J*) and *SLMBP24*] identified in tomato thus far. *J* is a member of the *StMADS11* subfamily (referred to as the SVP subfamily in Fig. 1), which also includes *Arabidopsis* SVP, AGL24, *Antirrhinum INCOMPOSITA* (*INCO*), and potato *StMADS11/16* (Carmona *et al.*, 1998; Garcia-Maroto *et al.*, 2000; Masiero *et al.*, 2004). The *StMADS11* subfamily is recognized for its broad range of functions. *J* was identified as a gene required for pedicel abscission zone development (Mao *et al.*, 2000). The mutation of *J* also affects inflorescence determinacy and to some extent flowering time (Szymkowiak and Irish, 1999, 2006; Quinet *et al.*, 2006).





**Fig. 1.** A Neighbor-Joining tree of MADS-domain proteins from tomato, petunia, and *Arabidopsis*. The representative MADS-box genes for major subfamilies are indicated on the right side of the tree and black filled circles represent the tomato MADS-domain proteins included in this study. Numbers along the branches are bootstrap values exceeding 50%. Subfamilies with <50% bootstrap support were collapsed. The *Arabidopsis* type I MADS-box protein PHERES was used as an outgroup.

In tomato, five members of the APETALA1/FRUIT-FULL (AP1/FUL) subfamily have been identified (Pnueli *et al.*, 1991; Vrebalov *et al.*, 2002; Hileman *et al.*, 2006). MACROCALYX (MC), TM4, SLMBP7, and SLMBP20 were chosen as representatives of this subfamily, with MC as an AP1 orthologue and the remaining three being FUL homologues. Of this group, only MC has been reported with functional data that indicated its role in calyx development and inflorescence meristem identity (Vrebalov *et al.*, 2002). TM4 was most similar to *Arabidopsis* ALG8/FUL, a gene that mediates cell differentiation during

*Arabidopsis* fruit development but also plays a role in floral meristem identity (Gu *et al.*, 1998; Ferrandiz *et al.*, 2000). In petunia, the FUL homologue PFG plays a crucial role in the transition from vegetative growth to inflorescence identity (Immink *et al.*, 1999). Unlike *Arabidopsis*, tomato has two AP3-like proteins, TAP3 (euAP3 lineage) and TM6 (TM6 lineage; Pnueli *et al.*, 1991; Kramer *et al.*, 1998), and two PI-like proteins, TPI and LePI which represent B-class function (de Martino *et al.*, 2006; Hileman *et al.*, 2006). The duplication of the two B-class MADS-box genes in tomato provided the possibility of novel protein interactions when compared with *Arabidopsis* B-class proteins. The AGAMOUS (AG) subfamily was represented by all four members TAG1, TAGL1, TAGL11, and SLMBP3 in the present assays. Phylogenetic evidence suggests that these proteins were homologues of *Arabidopsis* AG, SHATTERPROOF (SHP 1/2), and two different homologues of SEEDSTICK (STK), respectively (Fig. 1).

In addition, five tomato SEP subfamily proteins, TM5, RIN, TM29, LeMADS1, and SLMBP21, were used in the present study to compare not only their direct binding capabilities but also their bridging capabilities in higher order complexes. The *Arabidopsis* SEP3 protein and its orthologues have proven useful in interaction studies identifying higher order complexes among MADS-domain proteins (Favaro *et al.*, 2003; Ferrario *et al.*, 2003; Shchennikova *et al.*, 2004; de Folter *et al.*, 2006; de Martino *et al.*, 2006). Phylogenetic analysis clearly suggested TM5 as the orthologue of the *Arabidopsis* SEP3 (Fig. 1).

Constructs were designed to include the M, I, K, and C domains, and in some cases the C-terminus was partially truncated (see Supplementary Fig. S1 at *JXB* online; see Materials and methods). In addition, 16 genes were cloned without the MADS-box to test the role of their MADS-domain in protein interaction specificity.

#### Direct interactions of tomato MADS-domain proteins

All 22 tomato MADS-box genes were cloned in both pGAL-BD and pGAL-AD for a reciprocal mating scheme (Table 1). The SOC1 members showed a variable interaction network. TM3, for example, had an extensive interaction network that included all subfamilies with the exception of the B-class proteins. Similar to *Arabidopsis* SOC1, TM3 also formed a homodimer. SLMBP18 showed fewer interactions with the SEP-like and FUL-like proteins, and no interactions with the AG subfamily were observed. One protein in this subfamily, SLMBP14, failed to show any interactions with other proteins tested.

The SVP subfamily members J and SLMBP24 had a similar network, interacting with proteins from all subfamilies except the B-class proteins. Both showed a strong relationship with all five proteins from the SEP subfamily in a reciprocal manner. In contrast, interactions

with the AG and AP1/FUL subfamilies were mostly detected in a single direction. J and SLMBP24 were able to form homodimers and, despite the closely related interaction networks, they were unable to interact as a heterodimer. Direct interactions were not found between proteins representing the classic ABC functions for organ development such as MC, TAP3/TM6/LePI/TPI, and TAG1. The putative AP1 orthologue MC had a rather limited interaction network. MC, as well as the remaining three FUL-like proteins, was able to interact with the SEP protein RIN. The FUL-like proteins (TM4, SLMBP7, and SLMBP20), however, distinguished themselves by interacting with the SOC1-like protein TM3 whereas MC could not. All four proteins failed to homodimerize and were unable to interact with other members of the AP1/FUL subfamily.

B-class proteins TAP3 and TM6 showed a single interaction with a different member of the PI subfamily, i.e. TAP3 interacted with LePI whereas TM6 interacted with TPI (Table 1). LePI distinguished itself from TPI by interacting with two SEP-like proteins RIN and SLMBP21. All four B-class proteins failed to form homodimers, a phenomenon consistently observed in other eudicots studied (Winter *et al.*, 2002). The *Arabidopsis* AG orthologue TAG1 interacted with proteins in the SOC1 and SVP subfamilies, including TM3, J, and SLMBP24. TAG1 and TAGL1 demonstrated similar interaction patterns and distinguished themselves from TAGL11 and SLMBP3 by being able to interact with TM29 and TM3. The entire AG subfamily interacted with SLMBP21, RIN, and TM5 while showing a reduced relationship with TM29 and LeMADS1. The ability to interact with LeMADS1 and SLMBP7, which represent the SEP and FUL subfamilies, respectively, rendered TAG 1 a unique member among tomato AG-like proteins. Therefore, despite the additional B-class proteins in tomato, the main interaction of floral organ identity proteins remains dependent upon higher order complexes.

#### Tomato SEP-like proteins play unequal roles in the formation of higher order complexes

The *Arabidopsis* SEP proteins can directly interact with many MADS-domain proteins and have been shown to act as bridges for higher order complexes (Honma and Goto, 2001; Kaufmann *et al.*, 2005). In tomato, as in other plant species studied thus far, the interaction network involving SEP-like proteins was extensive (Table 1). All but four proteins, SLMBP14, TAP3, TM6, and TPI, were found to interact with at least one SEP-like protein directly.

For ternary complex assays, all five SEP-like genes were cloned into the pTFT1 vector and tested with all bait and prey clone combinations that failed to interact in Y2H assays (see Materials and methods). Tomato SEP-like proteins displayed variable capabilities in bridging ternary

**Table 1.** Interactions of MIKC tomato MADS-domain proteins

MIKC MADS-domain protein interactions as detected by the yeast two-hybrid system. The BD (bait) are listed along the vertical and the AD (prey) along the horizontal. Black and grey shading represent a reciprocal and one-way interaction, respectively. A failure to interact is indicated by a blank cell.

Phylogenetic subfamily	Bait	TM3	SLMBP18	SLMBP14	JOINTLESS	SLMBP24	MC	TM4	SLMBP20	SLMBP7	TAP3	TM6	LePI	TPI	TAG1	TAGL1	TAGL11	SLMBP3	SLMBP21	RIN	TM5		
SOC1																							
SVP/AGL24																							
AP1/FUL																							
AP3/PI																							
AG																							
SEP																							

interactions. TM5 exhibited a versatile role in bringing together multiple protein combinations, whereas the remaining SEP-like proteins appeared to play rather limited roles in ternary complex formation (Table 2). The AP1 orthologue MC and B-class protein LePI were both able to form complexes with TAG1, TAGL11, and SLMBP3 via TM5 (Table 2; see Supplementary Fig. S2 at *JXB* online). Interestingly, the MC bait and TFT:TM5Δ225 alone were able to activate the Y3H system (see Supplementary Fig. S2 at *JXB* online). Although the two proteins did not interact in the Y2H assay, they might actually form a heterodimer as AP1 and SEP3 do in

**Table 2.** Higher order complexes of tomato MIKC MADS-domain proteins

Yeast three-hybrid assays were used to detect putative MIKC MADS-domain complexes. The bait (BD), prey (AD), and bridge (pTFT1) are listed, with a '+' indicating a positive and a '-' indicating a negative interaction on selective media lacking Trp, Leu, His, and Ade supplemented with no less than 3 mM 3-AT.

pBD-GAL4	pTFT1	PAD-GAL4	Interaction	Complex
LePI	TAP3	TM5	+	B-B-E
	TAP3	Empty	-	
LePI	TM5Δ225	TAGL11	+	B-E-D
		SLMBBP3	+	B-E-D
		TAG1	+	B-E-C
		Empty	-	
TM4	TM5Δ225	SLMBP24	-	
	RIN	SLMBP24	+	FUL-E-FT <sup>c</sup>
	MADS1	SLMBP24	+	FUL-E-FT <sup>c</sup>
	RIN	Empty	-	
	MADS1	Empty	-	
TM4	Empty	SLMBP24	- <sup>a</sup>	
MC	TM5Δ208	TAGL11	+	AP1-E-D
		SLMBBP3	+	AP1-E-D
		TAG1	+	AP1-E-D
		Empty	- <sup>b</sup>	
TAG1	TM5Δ208	TAGL11	+	C-E-D
		TAGL1	+	C-E-D
		SLMBP3	+	C-E-D
		Empty	-	
TAGL1	TM5Δ208	TAGL11	+	D-E-D
		TAGL1	+	D-E-C
		SLMBP3	+	D-E-D
		TAG1	+	D-E-C
		Empty	-	
TAGL11	TM5Δ225	TAGL11	+	D-E-D
		TAG1	+	D-E-C
		SLMBP3	+	D-E-D
		TM3	+	D-E-SOC1
		Empty	-	
SLMBP3	TM5Δ208	TAGL11	+	D-E-D
		TAGL1	+	D-E-D
		TM3	+	D-E-SOC1
		Empty	-	

<sup>a</sup> TM4/SLMBP24 heterodimer is indicated in Table 1; however, the interaction was not positive on media supplemented with  $\leq 3$  mM 3-AT.

<sup>b</sup> MC/TM5/empty was positive using TFT:TM5 MIKCΔ225 which would suggest that MC and TM5 interact, although this was not observed in the yeast two-hybrid data.

<sup>c</sup> FT (flowering time) is being used in respect to the functional role of SLMBP24's *Arabidopsis* homologue AGL24.

*Arabidopsis* (Pelaz *et al.*, 2001). Complexes of B-class proteins with those of the SEP and AG subfamilies have been reported in *Arabidopsis*, petunia, and *Chrysanthemum* (Honma and Goto, 2001; Ferrario *et al.*, 2003; Shchennikova *et al.*, 2004). The AG subfamily proteins also showed TM5-dependent interactions among themselves as well as with the SOC1-like protein TM3. The various combinations of the putative tomato C- and D-class proteins with TM5 were analogous to the interactions of AG, SHP1/2, and STK with SEP3 in *Arabidopsis* (Favaro *et al.*, 2003).

Because several B-class proteins showed a rather limited interaction capability, it was of interest to determine whether the network could be expanded by first forming a B-class dimer. Therefore, TAP3 was cloned in the pTFT1 vector and combined with BD:LePI, BD:TM6, and BD:TPI, respectively. Yeast cells harbouring BD and TFT constructs were then screened against all available AD constructs. As shown in Table 2, the BD:LePI/TFT:TAP3 heterodimer was required for the formation of a ternary complex with AD:TM5. However, such a combination did not work for the second AP3-like protein TM6 and the PI-like protein TPI.

#### Use of IKC constructs altered protein-protein interaction patterns

Although large-scale yeast Y2H analysis in *Arabidopsis* and petunia were carried out using MIKC constructs, recent practices of utilizing IKC constructs continue to be reported (de Martino *et al.*, 2006; Cseke *et al.*, 2007). To gain insight into the effect of the MADS-domain on protein-protein interactions, 16 IKC constructs were developed. Compared with the interaction patterns derived from full-length proteins, most proteins IKC constructs exhibited a dramatic increase in interacting partners (Table 3).

The interaction networks of MC and TM4 IKC constructs were extensive and highly similar to one another. The clear difference between these two constructs was the ability of MC to interact with all three B-class proteins used. The B-class and AG-like proteins also exhibited a significant increase in interaction partners with proteins from all subfamilies.

To assess the role of the MADS-domain for ternary complex formation, IKC SEP-like constructs were used in Y3H assays to test their ability to bridge originally non-interacting proteins. In contrast to the bias given to TM5 in the full-length assays, all five SEP-like proteins displayed similar capabilities in ternary complex formation. As shown in Supplementary Table S2 at *JXB* online, 14 homo- and hetero-combinations that failed to interact in Y2H assays were now able to form ternary complexes in the presence of a SEP-like protein bridge. The overall trend of increased interaction partners and the loss of the specificity of IKC SEP-like proteins for ternary complexes

**Table 3.** Comparison between the MIKC-MIKC and IKC-IKC protein interaction networks

Yeast two-hybrid assays were performed using MIKC and IKC constructs and the data was pooled to demonstrate the difference between the two methodologies. The black and grey shading represents the MIKC- and IKC-specific interactions, respectively, while the grid pattern represents a positive in both assays. IKC BD:SLMBP24 and BD:SLMBP21 constructs were not available, 'n', for IKC assays.

Phylogenetic subfamily	Prey	TM3	JOINTLESS	SLMBP24	MC	TM4	TAP3	TM6	LePI	TAG1	TAGL1	TAGL1	TM29	SLMBP21	MADS1	RIN	TM5	
SOC1	Bait																	
	TM3																	
	JOINTLESS	Black	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid	Grid
SVP	SLMBP24	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	MC	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	TM4	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
AP1/FUL	TAP3	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	TM6	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	LePI	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
AP3/PI	TAG1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	TAGL1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	TM29	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
AG	SLMBP21	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	MADS1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	RIN	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
SEP	TM5	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	MADS1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	RIN	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n

demonstrated the importance of the MADS-domain in interaction assays.

## Discussion

Tomato distinguishes itself from *Arabidopsis* and petunia with its fleshy fruits and complex ripening processes which involve MADS-box genes (Pnueli *et al.*, 1991, 1994). To date, the total number of MIKC<sup>c</sup>-type tomato MADS-box genes has reached 36 (Hileman *et al.*, 2006). A total of 22 proteins were selected representing the AP1, AP3/PI, SVP, SOC1, AG, and SEP subfamilies of the MADS-box gene family that have been heavily studied in model plants. Each protein was tested for its ability to form dimers and higher order complexes with the rest of the proteins. The results showed that although interactions among essential MADS-domain proteins were conserved in tomato, divergences in interaction patterns were evident.

### Interaction patterns of members of the SVP/StMADS11 subfamily in tomato

The StMADS11 subfamily, named after the potato MADS-box gene *StMADS11*, contains genes of the most versatile functions, including the tomato *JOINTLESS/SLMBP24*, the *Arabidopsis SVP/AGL24*, potato *StMADS11/16*, and the *Antirrhinum INCOMPOSITA (INCO)*. These genes confer different functions in their respective species. For example, *INCO* in *Antirrhinum* is a suppressor of prophyll development along with a complex role in both floral repression and positive influence on floral meristem identity (Masiero *et al.*, 2004), whereas the loss of *SVP* shows severe early flowering but with normal flower development (Hartmann *et al.*, 2000). The overexpression of both *SVP* and *INCO* in *Arabidopsis* shows a similar phenotype with delayed flowering and leaf-like flowers (Masiero *et al.*, 2004). In tomato, *J* is most notable for its role in abscission zone development. Loss of *J* also results in the loss of inflorescence determinacy, which shows a conversion of floral to vegetative growth (Szymkowiak and Irish, 1999).

The protein interaction network of *J* was to some extent similar to that of *SVP*. *J* forms heterodimers with putative flowering time proteins SOC1-like TM3 and SLMBP18 as well as the AP1 orthologue MC and all of the SEP-like proteins (Table 1). This suggests putative roles for *J* in flowering time control or perhaps some aspects of floral organ development since all these genes are expressed throughout the flower (Vrebalov *et al.*, 2002; Hileman *et al.*, 2006). However, flowering time is only slightly decreased in *jointless* plants, and no floral abnormalities are present (Quinet *et al.*, 2006). This may be expected since cultivated tomato has experienced extensive domestication with a preference for fruit selection and potentially causing a loss of other traits such as flowering time



control (Quinet and Kinet, 2007). Although *INCO* over-expression was able to delay flowering in *Arabidopsis*, *inco* plants fail to display any changes in flowering time in *Antirrhinum* (Masiero *et al.*, 2004). The ability of *INCO* to homodimerize and autoactivate reporter genes in yeast is in contrast to *SVP* which can do neither. Interestingly, *J* was able to form a homodimer but lacks the autoactivation in yeast. This may suggest a different role for *J* in tomato as its functional complexes would require other MADS-domains protein(s) capable of activating transcription.

Although the interactions of SEP- and AP1-like proteins with putative *J* orthologues were conserved in *Arabidopsis* and *Antirrhinum*, new interactions may suggest that they have taken on novel roles in each species. For example, *J* was able to interact with all the members of the AG subfamily, an association observed for the SLMBP24 homologue AGL24 but not for *SVP*. Although *SVP* lacks such interactions, *INCO* was able to interact with C-function PLE, and *INCO* is expressed in mature stamens. *J* is expressed throughout the vegetative phases and in floral tissues. This includes the roots where other MADS-box genes, such as SLMBP20 and TM3, are expressed and which can interact with *J* (Mao *et al.*, 2000; Table 1). *In situ* hybridization identified *J* expressed in stamen and carpel primordia but later it became localized to the sporogenous tissue and later to the internal cells of the anthers and ovules (Szymkowiak and Irish, 2006). Thus, the interactions of *J* with putative C- and D-function proteins are in agreement with its expression pattern. However, there are no obvious developmental defects in the inner whorls of *jointless* plants. A number of interactions among the *StMADS11* members appear conserved among the species. The diverse functions among the *StMADS11* members could therefore be the result of an altered role for the same conserved complex that is involved in different biological processes in each species.

#### *The direct interaction of the tomato B-class and SEP proteins*

Unlike *Arabidopsis*, tomato has two sets of paralogues of both the AP3 and PI lineages, which is similar to the case of petunia. Recent studies in petunia and tomato, both in the Solanaceae, have reported on differential expression patterns and subfunctionalization of AP3 (euAP3) and TM6 paralogues in the respective species (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). In tomato, *TM6* plays partially redundant roles with *TAP3* in regard to floral development. However, the two genes have acquired distinct functions in tomato. The selective formation of B-class dimers (LePI/TAP3;TPI/TM6) is in line with their functional divergence. In petunia, the two PI proteins pMADS2 and FBP1 are able to interact with AP3-like pMADS1, while PhTM6 interacted only with pMADS2 (Immink *et al.*, 2003; Vandebussche *et al.*, 2004).

LePI distinguished itself among the B-class proteins for its ability to form higher order complexes as well as directly interacting with SEP-like proteins. Direct interaction of a B-class protein with the SEP subfamily was first demonstrated using MADS-deleted constructs and was later shown to be the case in *Chrysanthemum* using full-length constructs (Shchennikova *et al.*, 2004; Yang and Jack, 2004). Such an interaction in tomato would be the second report, which appears to be specific to LePI and not for the remaining B-class proteins. As suggested by Schennikova *et al.* (2004), the heterodimer of B/SEP proteins may actually represent a B-class protein homodimer interacting with the SEP protein in a higher order complex. However, as many studies in eudicots indicate, the B-class proteins fail to form homodimers in Y2H assays. LePI formed complexes with TAGL11 and SLMBP3, which represent homologues of FBP7 and FBP11 that play roles in seed and ovule development (Table 2; Angenent *et al.*, 1995; Colombo *et al.*, 1995). However, the expression patterns of tomato's two putative D-class proteins would not necessarily overlap with classic B-class proteins. Nevertheless, FBP24, a petunia B-sister protein, has been demonstrated to have a role in ovule development and to be able to form complexes with C and D function proteins (de Folter *et al.*, 2006). Also note that in IKC assays TAP3 was found to form a complex with TAGL11 via multiple SEP bridges (see Supplementary Table S2 at *JXB* online). Whether or not this is suggestive of a functional B/SEP/D complex in tomato awaits further experimentation.

#### *Interaction patterns of members of the AP1/FUL subfamily in tomato*

In comparison with *Arabidopsis*, the FUL subfamilies are expanded in both tomato and petunia. While the *Arabidopsis* genome has only one *FUL* gene, petunia and tomato have three and four members identified, respectively. Such gene copy amplification was apparently favourable for the plants and manifested in the increase of the interaction complexity in tomato. Among the three tomato FUL-like proteins, SLMBP7 was able to interact with nine proteins (TAG1, RIN, TM5, MADS1, SLMBP21, TM3, SLMBP18, J, and SLMBP24), far exceeding that of the other two FUL-like proteins SLMBP20 and TM4 which interacted with four and three, respectively (Table 1). Such a difference in interacting partners among FUL-like proteins did not occur in petunia in which the four FUL-like members show roughly equal interacting capabilities (Immink *et al.*, 2003). Tomato distinguishes itself from *Arabidopsis* by the interactions of FUL-like proteins with JOINTLESS. All three tomato FUL-like proteins fail to interact with TM29, whereas *Arabidopsis* FUL and petunia FBP29 and PFG show interactions with corresponding TM29 homologues.



Interestingly, petunia FUL-like proteins form heterodimers with each other (PFG/FPB29, FPB29/FPB26; Immink *et al.*, 2003), while such dimers did not exist in tomato, clearly indicating the functional divergence of FUL-like proteins in these two Solanaceae members. FPB29 also interacts with D-function FPB11, an occurrence not detected for the *Arabidopsis* and tomato counterparts. Since genes in the FUL subfamilies have been shown to be involved in fruit and ovule development (Gu *et al.*, 1998; Ferrandiz *et al.*, 2000), the divergence in protein interaction patterns may be responsible for developmental divergence of fruits in these species.

#### *Interaction patterns of functionally divergent SEPALLATA proteins in tomato*

Among the five SEP-like proteins in tomato (Malcomber and Kellogg, 2005; Hileman *et al.*, 2006), the most distinguishable is RIN, which plays an important role in tomato fruit ripening (Vrebalov *et al.*, 2002). RIN was able to interact with all members of the AP1/FUL and AG subfamilies, which have been shown to be essential in silique development in *Arabidopsis* (Gu *et al.*, 1998; Liljegren *et al.*, 2000). Although it did not play a major role in ternary complex formation, RIN interacted with multiple putative flowering time proteins in a direct manner, suggesting additional roles in tomato flower development. Oddly, *rin* mutants fail to show any obvious abnormalities beyond fruit ripening, suggesting that interactions are functionally redundant or potential false positives. Existing expression data indicate that RIN is predominantly located in fruit tissue (Hileman *et al.*, 2006). As shown in Supplementary Table S3 at *JXB* online, nine of RIN's interactions would not be spatially possible with the current available expression data. However, RIN transcripts have been detected in both young buds and mature flowers, indicating its expression in these tissues to some extent (data not shown). In contrast to the single mutations of *Arabidopsis* SEP genes, the mutation of one tomato SEP gene often causes dramatic phenotypic changes. For example, down-regulation of *TM29* produced *sepallata*-like flowers, parthenocarpic fruits, and ectopic shoots (Ampomah-Dwamena *et al.*, 2002). A *TM5* antisense transgene resulted in floral organ abnormalities in the third and fourth whorls (Pnueli *et al.*, 1994). Such functional specificity and lack of redundancy is in contrast to the cases in *Arabidopsis* and suggests that the functions of tomato SEP proteins are more diverged and may be associated with proteins working in different genetic pathways. The interpretation of *TM29* and *TM5* phenotypes, however, should be made with caution as they were both generated using antisense cDNA expression approaches and it is still unclear whether multiple similar SEP genes were indeed affected in these experiments. In petunia, overexpression of *FBP2*

was able to co-suppress *FBP5*, a close homologue, leading to significant floral abnormalities (Ferrario *et al.*, 2003). Subsequent work determined that single knock-out of *FBP2* altered floral development whereas plants with a single mutation of *FBP5* showed wild-type flowers. (Vandenbussche *et al.*, 2003). The other two SEP genes *LeMADS1* and *SLMBP21* appear to be paralogues based on phylogenetic analysis (Fig. 1). The two genes shared nearly identical expression patterns, with transcripts detected in inflorescence, floral organs, and fruits (Hileman *et al.*, 2006), but with different protein interaction patterns that may underline their potential functional divergence.

Tomato SEP proteins could interact with each other by forming homo- and heterodimers as has been reported in other protein interaction studies (Immink *et al.*, 2003; de Folter *et al.*, 2005). Various combinations of SEP interactions were abundant in tomato but most notably missing were those of *TM5*. *TM5*, however, showed exceptional capability in forging ternary complexes. It was a consistent bridge for ternary complexes of AG-like proteins, a phenomenon also observed in *Arabidopsis* (Favaro *et al.*, 2003). *TM5* was also able to bridge the interactions between MC and members of the AG subfamily. MC is expressed in the carpel (Vrebalov *et al.*, 2002), similar to the *Antirrhinum* AP1 orthologue *SQUA* which also interacts with two C-function proteins FARINELLI and PLENA (Davies *et al.*, 1996).

Also interesting is that RIN and *LeMADS1* bridged the same two proteins, *TM4* and *SLMBP24*, in ternary complexes (Table 2; see Supplementary Fig. S2 at *JXB* online). The clear function of *TM4* has not been defined in tomato; however, its homologue in *Arabidopsis*, *FUL*, mediates cell differentiation during fruit development and also plays a role in floral meristem identity (Gu *et al.*, 1998; Ferrandiz *et al.*, 2000). *SLMBP24*, similarly to *AGL24*, is primarily expressed in non-floral tissues, suggesting that the *TM4*/SEP/*SLMBP24* complexes may play certain roles in floral meristem identity rather than fruit development.

#### *Interaction patterns of putative orthologous MADS-domain proteins in tomato, Arabidopsis, and petunia*

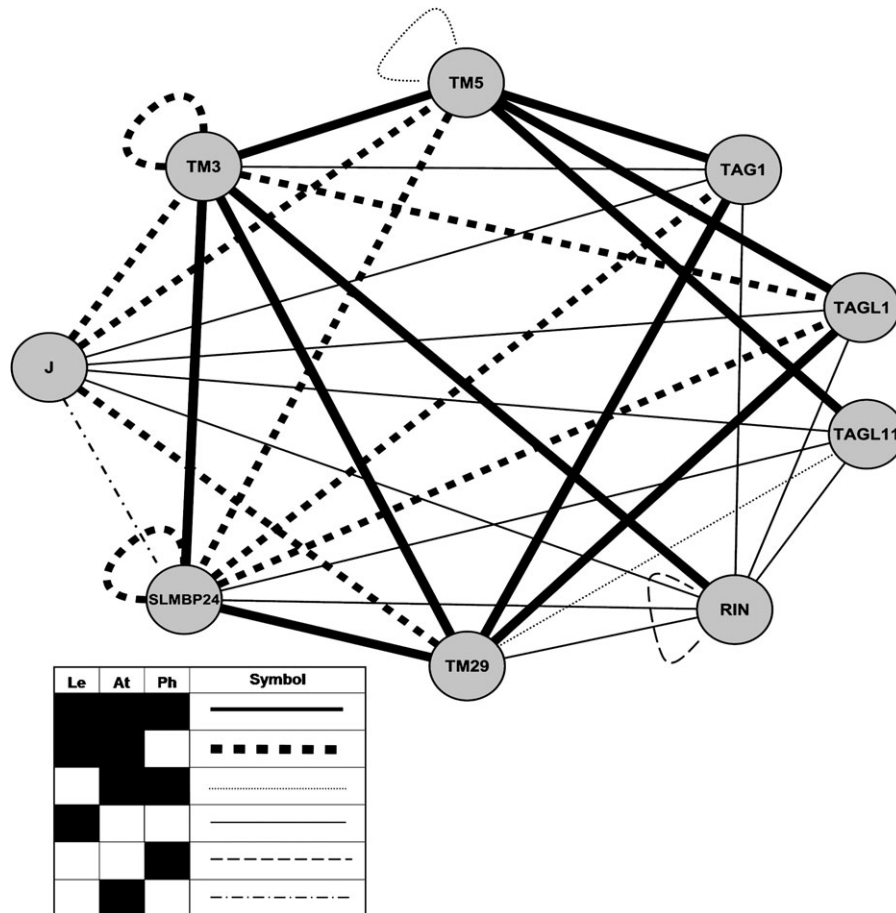
Although the MIKC<sup>c</sup>-type MADS-box genes have undergone extensive duplications, a high degree of protein interaction conservation has been retained throughout evolution (Veron *et al.*, 2007). In most cases, tomato MADS-domain proteins exhibited similar interaction patterns to their counterparts in *Arabidopsis* and petunia. However, deviation of interaction patterns was also observed and may well represent functional divergence. A composite figure was generated to give an impression of overall conservation and divergence of MADS-domain protein interaction networks among the three species in which a large-scale protein interaction analysis has been

conducted (Fig. 2). Among the most conserved interactions, orthologues of TM5 were able to interact with AG-like proteins (nodes represented by TAG1, TAGL1, and TAGL11) in all three species. Interestingly, tomato and *Arabidopsis* share more connected nodes when an interaction is not conserved among all three species, such as the SLMBP24 node which interacts with nodes represented by TM5, TAG1, and TAGL1 that are absent in petunia. In contrast, the two orthologues represented in the J node show greater interaction divergence between *Arabidopsis* and tomato, with the tomato J interacting with four nodes that are not present for the *Arabidopsis* SVP. It should be noted that a significant proportion of the petunia interaction data comes from assays that were performed at 37 °C. It is possible that some interactions were not stable at this temperature and thus not detected by the authors (Immink *et al.*, 2003). Therefore, the discrepancy observed between tomato and petunia may be

a result of the different methods by which the interactions were detected. The TM3 node has a conserved connection with the three SEP nodes as well as the SLMBP24 node, which may represent a conserved mechanism for flowering time control or flower meristem identity in the three species. The TM3 node also interacts with the J node for *Arabidopsis* and tomato. Nevertheless, TM3 shows a tomato-specific interaction with TAG1. The RIN node distinguishes itself by showing increased numbers of tomato-specific interactions by connecting with all three AG-like nodes, a trend not observed for SEP4 and FBP4 from *Arabidopsis* and petunia, respectively.

#### The impact of the MADS-domain on protein–protein interactions

It has been suggested that the MADS-domain, being a highly conserved DNA-binding domain, could



**Fig. 2.** Interaction network of putative orthologous MADS-domain proteins from *Arabidopsis*, petunia, and tomato. Putative orthologues were the closest homologues derived from phylogenetic analysis. Malcomber and Kellog (2005) was referenced for the formation of the TM29 SEP node. The nodes were named using tomato MADS-domain proteins. Each node represents putative orthologous proteins from two or three species: TM5 [TM5;SEP3;FBP2], TAG1 [TAG1;AG;PMADS3], TAGL1 [TAGL1;SHP1/2;FBP6], TAGL11 [TAGL11/SLMBP3;STK;FBP7/11], RIN [RIN;SEP4;FBP4], TM29 [TM29;SEP1/2;FBP5;pMADS12], SLMBP24 [SLMBP24;AGL24;FBP13], TM3 [TM3;SOC1;FBP21], and J [J;SVP;n]. *Arabidopsis* (A), tomato (T), and petunia (P). Node edges represent the following: ATP [thick solid line], AT [thick/short dashed line], AP [dotted line], A [uneven dashed line], P [thin/long dashed line], T [thin solid line].

potentially cause activation of reporter genes by binding to their promoter region in the Y2H system. However, this would require the MADS-domain to bind to the GAL4 UAS of the yeast reporter genes, which has not been formally addressed in any publication. The present results showed that the use of IKC, rather than constructs containing the MADS-domain, may yield false positives. The comparison of the two cloning approaches showed that the inclusion of the MADS-domain, in most cases, decreased the number of interactions. The present study helps to bring awareness to the possible false positives generated by protein constructs lacking the MADS-domain.

## Conclusion

Although prone to false positives, Y2H analyses provide the first insight into protein interaction possibilities and thus their potential functional modes. Available expression data showed that all but nine interactions in this study could occur in the same tissue, thus providing spatial possibility for these interacting proteins (see Supplementary Table S3 at *JXB* online). The current work adds to the growing body of interaction information focused on the MADS-domain transcription factors. The availability of this family's interaction network in tomato provides a basis for further functional and evolutionary study of this important gene family. The present results on the role of the MADS-domain in protein interactions may stimulate further consideration in future experimental design involving MADS-domain proteins.

## Supplementary data

Supplementary data are available at *JXB* online.

**Table S1.** MIKC MADS-domain protein interactions at varying levels of stringency.

**Table S2.** Higher order complexes of tomato IKC MADS-domain proteins.

**Table S3.** Co-expression of genes whose proteins interact in yeast two-hybrid assays.

**Fig. S1.** Tomato MADS-domain proteins constructs used in the yeast two-hybrid assays.

**Fig. S2.** Subsets of tomato MADS-domain yeast two- and three-hybrid assays.

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