In situ hybridization of the MADS-box gene POTM1 during potato floral development

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Abstract

The potato MADS-box gene POTM1 is a member of the SQUA-like family of plant MADS-box genes. The amino acid sequence of POTM1 most closely matches the sequence of PFG (Petunia Flowering Gene), a petunia MADS-box gene involved in the transition from vegetative to reproductive development. To examine the likely role of POTM1 in potato reproductive tissue, in situ hybridizations were performed on sections of the potato shoot apex at various stages during the transition to flowering. Scanning electron micrographs provide a reference for each stage to illustrate the progression from vegetative meristem to inflorescence meristem and floral organs. POTM1 mRNA accumulates in vegetative, inflorescence and floral meristems. Transcripts are present in petal primordia during the later stages of petal development and they accumulate later during floral development inside the stamens and carpels, but not in carpel walls. This widespread pattern of mRNA accumulation is unique among the SQUA-like genes and indicates that POTM1 is active during a variety of stages of development, and may function in regulating cell determination in transitional phases of both vegetative and floral meristems.

Key words: Floral development, homeotic gene, MADS-box, potato.

Introduction

During plant development and floral organogenesis in particular, genes of the MADS-box family function in key regulatory roles (reviewed in Ng and Yanofsky, 2001). Consequently, these genes have been extensively studied in a variety of plant species. MADS-box genes contain a conserved region which codes for a DNA-binding domain, termed the MADS-box, which enables the products of the genes to act as transcription factors controlling the formation of organs and the induction of developmental stages (Rounsley et al., 1995; Riechmann and Meyerowitz, 1997). Many MADS-box genes are involved in controlling the formation of the floral organ whorls according to the ABC model of development (Bowman et al., 1991; Pelaz et al., 2000). Others have been implicated in ovule development (Angenent et al., 1995), floral meristem determinacy (Huijser et al., 1992), and in the transition from vegetative to reproductive phases of growth (Mandel and Yanofsky, 1995; Immink et al., 1999; Borner et al., 2000; Elo et al., 2001). Many of the MADS-box genes have multiple roles in several stages of development. APETALA1 (AP1), for example, is a gene which is instrumental in floral meristem determination, and later controls sepal and petal identity (Mandel et al., 1992).

MADS-box genes have been classified based on both sequence identity and expression pattern, and separated into several distinct functional groups (Theißen et al., 1996). The SQUAMOSA-like genes are named after the SQUAMOSA (SQUA) MADS-box gene from Antirrhinum majus (Huijser et al., 1992). This gene controls floral meristem identity, and recessive mutants lose the ability to maintain floral meristems. Meristems are induced to form in bract axils, but without SQUA expression, they form shoots instead of flowers. These shoots produce bracts, and the aberrant developmental process is repeated. In accordance with its mutant phenotype, SQUA is expressed in floral meristems of wild-type plants, but not in inflorescence or vegetative meristems. AGL8 (FUL) is a MADS-box gene in Arabidopsis thaliana which shares a close sequence match with SQUA (Mandel 1 To whom correspondence should be addressed. Fax: +1 515 294 0730. E-mail: djh@iastate.edu

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and Yanofsky, 1995). It is expressed in the inflorescence meristem, but not in vegetative meristems, and has been implicated in cell differentiation during fruit development (Hempel et al., 1997; Gu et al., 1998; Ferrandiz et al., 2000). The Petunia Flowering Gene (PFG) has a 64% identity match to AGL8 and is expressed throughout the plant except in roots and stamens (Immink et al., 1999). Suppression mutants of PFG produce a unique, non-flowering phenotype. Genetic analyses indicated that PFG is an inflorescence meristem identity gene that acts early to control the transition to flowering.

**POTM1** is a SQUA-class MADS-box gene from potato (Kang and Hannapel, 1995) that shares a very close sequence match with PFG. POTM1 is expressed at the RNA level in vegetative tissues, including leaves, roots, and stolons, as well as in reproductive tissues. Several MADS-box genes, including AGL3 from Arabidopsis (Ma et al., 1991) and STMADS11 and STMADS16 from potato (Carmona et al., 1998), are expressed in vegetative organs, but the closest sequence matches to POTM1 belong to the SQUA-class of MADS-box genes, which function in floral development. To study the expression pattern of POTM1 further, *in situ* RNA hybridization was performed on potato floral organs at various stages of development. The accumulation of POTM1 mRNA occurs in vegetative, inflorescence and floral meristems; in petal, stamen, and carpel primordia; and in later stages of stamen and carpel development. This unique, widespread expression pattern, including vegetative and floral organs, indicates that POTM1 may be involved in both developmental phases.

**Materials and methods**

**Plant material**

Potato (*Solanum tuberosum* cvs Superior and Kennebec) plants were grown in a greenhouse at 25 °C under long-day light conditions (16:8 h day/night). 'Superior' shoot tips were harvested daily between 7 a.m. and 12 p.m. after shoot transplanting. Secondary shoot tips were also harvested at these times after removal of the original shoot apex. This span of time includes meristem development from the vegetative through later floral stages.

**In situ hybridization analysis**

Tissue samples were prepared as described previously (Cañas et al., 1994). Non-radioactive digoxigenin-labelled 0.7 kb POTM1 RNA lacking the conserved MADS-box region was used as a probe. Both sense and antisense RNA probes were transcribed with RNA polymerases according to the instructions of Boehringer-Mannheim, and hydrolysed by using 0.2 M sodium carbonate and 0.2 M sodium bicarbonate at 65 °C for 30 min. Unincorporated nucleotides were removed over a Sephadex G-50 column.

Slides with tissue sections were deparaffinized with xylene and hydrated through an ethanol series to 50 mM TE buffer pH 7.6, treated with 1 g ml−1 proteinase K in 50 mM TE buffer pH 7.6 for 30 min, washed with sterile deionized water three times for 10 min, treated with 0.25% acetic anhydride in 85 mM triethanolamine buffer pH 8.0 for 5 min, washed with sterile deionized water three times for 5 min, and dehydrated through an ethanol series to dry completely under vacuum. Hybridization took place in a humidified box at 45 °C with 0.6 µg ml−1 of the digoxigenin-labelled RNA probe specific for POTM1 in 50% formamide overnight. The slides were washed for 15 min in 2× SSC at 25 °C, three times in 0.2× SSC at 55 °C, treated with 20 µg ml−1 RNase A in 500 mM NaCl–TE buffer pH 8.0 at 37 °C for 30 min, washed three times in sterile deionized water, incubated in buffer 1 (1% blocking solution, 100 mM TRIS pH 7.5, 150 mM NaCl) for 1 h, then equilibrated with buffer 2 (100 mM TRIS pH 7.5, 150 mM NaCl, 0.5% BSA, and 0.3% Triton X-100). Tissue sections were then incubated with antidigoxigenin-alkaline-phosphatase conjugate diluted 1:1000 in buffer 2 in a humidified box for 2 h, then washed three times for 20 min in 100 mM TRIS pH 7.5, 150 mM NaCl. The tissue sections were equilibrated in buffer 3 (100 mM TRIS pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 10 min, then incubated in 3.2 µg ml−1 5-bromo-4-chloro-3-indolyl-phosphate (BCIP): 6.6 µg ml−1 nitro-blue tetrazolium salt (NBT) in buffer 3 in a humidified box for 13 h (above-ground tissues) or 7 h (below-ground tissues). Alkaline phosphatase produces a NBT-formazan precipitate which stains the cells containing an accumulation of POTM1 mRNA. The precipitate is viewed as an orange/brown stain under dark field illumination. Sections were viewed and photodocumented using dark field microscopy at 25× magnification on the Leitz Orthoplan light microscope.

**Scanning electron microscopy**

Tissue samples were fixed in 0.1 M cacodylate, 2% paraformaldehyde, 2% gluteraldehyde buffer, pH 7.2 under 15 psi vacuum for 24 h, then stored at 4 °C. Samples were washed with 0.1 M cacodylate buffer for 3× 10 min, then post-fixed in 0.1 M cacodylate, 1% OsO4 for 1 h. Samples were washed in 0.1 M cacodylate buffer for 10 min, and distilled water 2×10 min before dehydration over an ethanol series to 3×20 min washes in ultrapure ethanol. Samples were stored in ultrapure ethanol until critical point dry, with seven 2 min flushes alternated with 5 min rest periods. Leaves and several floral organs were removed after critical point drying for better viewing. Samples were coated with a Polaron E5100 SEM coating unit equipped with a Polaron E5200 coater. Samples were coated with a thin film of gold target (60%). Shoot apices were viewed under the JEOL 5800LV scanning electron microscope. Images were collected and stored digitally using ARC 58 image software (JEOL, Japan). Negatives were made of stored images using a Polaroid camera (665 film).

**Results and discussion**

**Sequence classification of POTM1**

Based on the most recent MADS domain protein sequences available, POTM1 has been placed in the SQUA-like family of MADS-box genes (Theißen et al., 1996). A comparison of amino acid sequences shows that the highest sequence similarity matches to POTM1 are all members of the SQUA-like family (Table 1). POTM1 shares only 50% similarity overall with AGL3 from Arabidopsis, which is expressed in vegetative tissue, but is not a member of the SQUA-like family (Ma et al., 1991). Besides the other *Solanum* species, POTM1 shares the
The inflorescence meristem on the right is larger than the one on the left, which is adjacent to the youngest leaf primordia. The leaf primordia are still identifiable in their spiral phyllotaxy, but in subsequent stages of potato inflorescence development, no more leaves or bracts are formed (Fig. 1A). The axillary bud forming between the smallest leaf primordia and the left side inflorescence meristem (Fig. 2B) will remain vegetative (Danert, 1957). Floral meristems arise from a cleavage of the inflorescence meristem into two apices, one floral and one inflorescence (Fig. 2C, c2). A stage 1 Arabidopsis floral meristem is characterized by the initiation of a floral buttress, corresponding to the initiation of the cleavage furrow in potato. In Fig. 2C, both inflorescence meristems have divided into an inflorescence and a floral meristem, but only the top left floral meristem has developed sepal primordia at this stage, corresponding to stage 3 of Arabidopsis floral development (Smyth et al., 1990). The lower half of the inflorescence, presented at greater magnification in Fig. 2D, has undergone cleavage (designated c3) to produce a stage 2 floral meristem, in which separation of the two meristems is distinct, but sepal primordia have not yet formed. In Fig. 2E, a greater magnification of the floral and inflorescence meristem pictured at the top of Fig. 2C, it is apparent that the inflorescence meristem has again undergone division, shown by a faint cleavage furrow (designated c4). In Fig. 2F, a stage 5 flower is shown, in which petal and stamen primordia have appeared, and the carpel will begin to emerge from the remnant of the central floral meristem visible within the flower. Sepals have been removed to facilitate viewing. Figure 2G shows a late stage 6 flower, with the developing carpel visible between two stamens. Sepals, petals, and two stamens have been removed to show the carpel. The scanning electron micrographs in Fig. 2 show the progression of the earliest stages of reproductive development in the potato plant, and provide points of reference for viewing the corresponding in situ sections of the shoot apex.

**Potato inflorescence development**

The first step in the transition from vegetative to inflorescence development in potato is the initiation of two determinate inflorescence meristems, produced by a cleavage of the shoot apical meristem (Danert, 1957). In most Solanum tuberosum L. plants, both inflorescence meristems continue to develop floral meristems, producing double, mirror-image scorpioid cymes (Fig. 1A). Under some conditions, the inflorescence meristems may double again before floral meristem initiation, producing four scorpioid cymes (Fig. 1B). Single cymes have also been observed. Although Arabidopsis inflorescence meristems are indeterminate, floral developmental stages are similar enough to characterize potato floral meristem development based on the corresponding stages in Arabidopsis (Smyth et al., 1990). Figure 2A shows the vegetative shoot apex of the potato plant before the transition. The apex is whole and forms a dome, around which leaf primordia originate in a spiral phyllotaxy. Figure 2B documents the transition from the vegetative to the reproductive phase with the initial cleavage (designated c1) of the apical meristem into two inflorescence meristems.

**Table 1. Comparison of the amino acid sequence similarity between POTM1 and other MADS-box proteins**

Overall percentages compare the entire amino acid sequence. MADS-domain percentages compare only the 57 conserved residues encoded by the MADS-box, whereas the K-box includes 53 aa of this conserved region. All proteins belong to the SQUA-like family. Similarity percentages were obtained using the GAP alignment program in GCG (Genetics Computer Group, Wisconsin Package Version 9.1, Madison, WI).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Percentage similarity</th>
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<tbody>
<tr>
<td></td>
<td>Overall K-box</td>
<td>MADS K-box</td>
</tr>
<tr>
<td>SCM1</td>
<td>S. commersonii</td>
<td>97 100 95</td>
</tr>
<tr>
<td>PFG</td>
<td>Petunia</td>
<td>91 100 89</td>
</tr>
<tr>
<td>TDR4</td>
<td>Tomato</td>
<td>87 100 95</td>
</tr>
<tr>
<td>NAP1-1</td>
<td>Tobacco</td>
<td>86 100 91</td>
</tr>
<tr>
<td>SLM5</td>
<td>Silene latifolia</td>
<td>76 98 86</td>
</tr>
<tr>
<td>AGL8</td>
<td>Arabidopsis</td>
<td>71 96 81</td>
</tr>
<tr>
<td>SQUA</td>
<td>Antirrhinum</td>
<td>71 95 80</td>
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<tr>
<td>API</td>
<td>Arabidopsis</td>
<td>69 95 81</td>
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The highest similarity with PFG from petunia: 91% overall and 100% within the MADS domain. Similar to POTM1, PFG transcripts were detected in both vegetative and floral organs (Immink et al., 1999). POTM1 shares 69% overall sequence similarity with API of Arabidopsis, and a 71% match to SQUA, both of which are expressed only in floral meristems. Within the SQUA-like family, POTM1 has a higher sequence match to proteins that exhibit a wide pattern of expression (PFG and SLM5, for example) than to those with a limited pattern of expression (API and SQUA, for example). These differences in sequence and patterns of expression may reveal a dichotomy of function within the SQUA-like family of genes.

**Potato MADS-box gene expression**
vegetative shoot apex (Fig. 3A, B), POTM1 mRNA is detected in the apical meristem, the procambium, and the lamina of older leaves and leaflets (Fig. 3B). POTM1 expression thus is not limited to the indeterminate apical meristem, but appears in cells which are fated to become leaves as well. Figure 3B corresponds to a longitudinal section through the apex shown in Fig. 2A. In a longitudinal section (Fig. 3C) through the shoot apex during the transition phase between vegetative and floral growth comparable to that shown in Fig. 2B, POTM1 accumulates in both inflorescence meristems, as well as in vascular tissue, axillary buds, and the lamina of young leaves. The POTM1 signal is less intense in the centre of the apex where cleavage is taking place (designated c1). POTM1 mRNA is also detected in the apical layers of floral meristems (Fig. 3D, E). The bulbous shape of the
meristems in Fig. 3D and the location of the subtending leaves indicate that this stage of development corresponds to that shown in Fig. 2D. At stage 2 of floral development, it is impossible to distinguish between floral and inflorescence meristems in potato; however, \textit{POTM1} accumulates equally in both. This pattern of expression is very similar to \textit{PFG}. Transcripts for \textit{PFG} were detected in the tunica and corpus layers of both vegetative and floral meristems and in the lamina and procambium of newly formed leaves (Immink et al., 1999).

In a stage 4 floral meristem (Fig. 3E) the sepal primordia have begun to overlie the floral meristem. \textit{POTM1} is again absent from the subset of apical cells which reside in the cleavage furrow. At this stage, just slightly more mature than the meristem pictured in Fig. 2E, \textit{POTM1} accumulates inside the floral meristem, but not in the sepal primordia. This is in contrast to the expression patterns of \textit{SQUA}, \textit{AP1}, \textit{SLM5}, and \textit{PFG}, all of which exhibit accumulation in the developing sepalas (Huijser et al., 1992; Mandel et al., 1992; Hardenack et al., 1994; Immink et al., 1999). In the stage 5 flower (Fig. 3F), \textit{POTM1} accumulates in petal and stamen primordia, as well as in the central remnant of the floral meristem which will eventually form the carpel. The \textit{in situ} section shown in Fig. 3F corresponds to the scanning electron micrograph shown in Fig. 2F. In the stage 6 flower (Fig. 3G), \textit{POTM1} transcript is evident in the carpel interior, but is absent from sepals, petals, and carpel walls. The absence of \textit{POTM1} transcript in carpel walls is in contrast to the expression patterns of \textit{AGL8} and \textit{SLM5} (Hardenack et al., 1994; Mandel and Yanofsky, 1995). Figure 3A shows the negative sense control for the vegetative shoot apex corresponding to Fig. 3B. Sense controls for all
The function of POTM1 in potato meristems

POTM1 mRNA has previously been shown to accumulate preferentially in actively growing vegetative organs (Kang and Hannapel, 1996). In this study, POTM1 mRNA is detected in vegetative, inflorescence and floral meristems, each of which are undergoing growth, but it is not detected at the cleavage furrow, an area which is relatively inactive compared to the meristems developing at either side. By stage 4, POTM1 expression is absent from the sepal primordia, which have already grown to cover the floral meristem. POTM1 transcripts were detected in actively growing petal primordia at stage 5, but not in stage 6, when petal primordia growth is slow. By contrast, POTM1 transcripts are detected in stamens and carpel interiors during stage 6, when these interior organs are growing more rapidly than petals (Smyth et al., 1990). Further insight into the function of POTM1 is provided by a comparison to the mutant phenotype of PFG (Imnink et al., 1999). Sequence similarity between POTM1 and PFG is 91%, and the RNA expression pattern of PFG is also nearly ubiquitous, including vegetative and reproductive meristems and differentiated organs. The mutant phenotype for PFG is characterized by a failure to initiate the reproductive phase of growth. Because of the close sequence similarity and expression pattern between POTM1 and PFG, they may have related functions in meristem transitional states. PFG is a meristem identity gene, which is essential for determining the identity of the inflorescence meristem during the switch from the vegetative to the reproductive phase of growth. POTM1 was originally isolated from an early tuberization stage cDNA library (Kang and Hannapel, 1995). This stage of growth is marked by an increase in cell growth and a change in the orientation of cell division in the subapical region of the stolon apex (Reeve et al., 1969). POTM1 transcripts were detected in actively growing cell types throughout tuberizing stolon tips (data not shown). It is possible that POTM1 may function in regulating cell determination in transitional phases of both vegetative and floral meristems.

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