Suppression of a Vegetative MADS Box Gene of Potato Activates Axillary Meristem Development

Faye M. Rosin, Jennifer K. Hart, Harry Van Onckelen, and David J. Hannapel*

Interdepartmental Program in Molecular, Cellular, and Developmental Biology, Department of Horticulture, Iowa State University, Ames, Iowa 50011–1100 (F.M.R., J.K.H., D.J.H.); and University of Antwerp, Universiteitsplein 1, B–2610, Antwerpen, Belgium (H.V.O.)

Potato MADS box 1 (POTM1) is a member of the SQUAMOSA-like family of plant MADS box genes isolated from an early stage tuber cDNA library. The RNA of POTM1 is most abundant in vegetative meristems of potato (Solanum tuberosum), accumulating specifically in the tunica and corpus layers of the meristem, the procambium, the lamina of new leaves, and newly formed axillary meristems. Transgenic lines with reduced levels of POTM1 mRNA exhibited decreased apical dominance accompanied by a compact growth habit and a reduction in leaf size. Suppression lines produced truncated shoot clusters from stem buds and, in a model system, exhibited enhanced axillary bud growth instead of producing a tuber. This enhanced axillary bud growth was not the result of increased axillary bud formation. Tuber yields were reduced and rooting of cuttings was strongly inhibited in POTM1 suppression lines. Both starch accumulation and the activation of cell division occurred in specific regions of the vegetative meristems of the POTM1 transgenic lines. Cytokinin levels in axillary buds of a transgenic suppression line increased 2- to 3-fold. These results imply that POTM1 mediates the control of axillary bud development by regulating cell growth in vegetative meristems.

During vegetative development, the processes of apical growth and lateral branching are important for determining patterns of growth in the shoot and inflorescence. The tremendous diversity in vegetative pattern formation between plant species arises from these same basic processes. Interactions between the shoot apical meristem (SAM), the axillary meristems, and signaling compounds transported from the roots are responsible for the overall pattern of shoot and branching architecture that is observed. Although these interactions are genetically determined, they are also mediated by internal and external cues such as hormone levels, light, or mechanical stimuli (for review, see Sussex and Kerk, 2001). The SAM is responsible for primary shoot growth, whereas lateral branching is initiated by the development of axillary meristems. Produced in the axils of leaves, axillary meristems arise postembryonically and are derived either directly from the meristematic cells of the SAM (potato [Solanum tuberosum]) or from cells on the adaxial surface of the subtending leaf (Arabidopsis; Sussex, 1955; Schmitz and Theres, 1999). There are two stages of axillary meristem development: initial formation and subsequent growth. After axillary meristem initiation, the SAM maintains its role as the primary site of growth by inhibiting the growth of axillary meristems. This phenomenon, called apical dominance, is mediated by auxin levels and putative second messengers that relay the auxin signal (Chafﬁeld et al., 2000). In potato, underground stems produce a specialized vegetative shoot called the stolon. The stolon meristem grows as a horizontal stem and will develop into a tuber under favorable conditions or a vegetative shoot if exposed to sufficient light.

A number of plant transcription factors play an important role in regulating the development of the SAM. MADS box genes are an example of a family of highly conserved transcription factors that have diverse roles in plant development. Although not examined as extensively as their role in floral organ identity (Weigel and Meyerowitz, 1994; Theissen, 2001), MADS box genes are important regulators of vegetative development. JOINTLESS, a tomato (Lyco-persicon esculentum) MADS box gene, is required for the development of a functional abscission zone in tomato flowers (Mao et al., 2000). PkMADS1 from the woody species Paulownia kawakamii is involved in controlling vegetative organ formation. Suppression of PkMADS1 in antisense transgenic plants disrupted the interaction between leaf primordia and the SAM, resulting in additional leaf formation with altered phyllotaxy at the expense of meristem maintenance (Prakash and Kumar, 2002). Transcripts of the potato MADS box genes STMADS11 and STMADS16 are present in all vegetative tissues of the potato including roots and new tubers but are not detected in floral organs (Carmona et al., 1998; Garcia-Maroto et al., 2000). Overexpression of STMADS16 in tobacco (Nicotiana tabacum) altered the architecture of the inflorescence, resulting in increased branching and internode length. Whereas flowers exhibited leaf char-
characteristics, the morphology of vegetative organs was not affected, indicating that STMADS16 promotes vegetative development (García-Maroto et al., 2000).

Members of the SQUAMOSA (SQUA)-like family of MADS box genes appear to have diverse roles in both vegetative and floral development including control of floral meristem and perianth organ identity (API; Mandel et al., 1992; CAL; Kempin et al., 1995), fruit morphogenesis (AGL8/FUL; Gu et al., 1998), and inflorescence identity (PETUNIA FLOWERING GENE [PFG]; Immink et al., 1999). Loss-of-function mutants of SQUA are characterized by the formation of shoots instead of flowers in the axils of bracts, indicating that SQUA is involved in the transition to reproductive development, thus controlling floral meristem identity in snapdragon (Antirrhinum majus; Huijser et al., 1992). Cosuppression of Antirrhinum floral meristem identity in snapdragon (SQUA)/1995), fruit morphogenesis (AGL8/FUL; Mandel et al., 1992; AP1/1995), a SQUA-like MADS box gene, leads to a non-flowering phenotype in which the vegetative phase is maintained. PFG acts earlier than SQUA, because pfg mutants are blocked in the vegetative to inflorescence transition, indicating that PFG is essential for inflorescence identity (Immink et al., 1999).

Potato MADS box 1 (POTM1) from potato was isolated from an early tuber cDNA library and belongs to the SQUA-like family of MADS box genes (Kang and Hannapel, 1995). POTM1 has the highest sequence match to SCM1 from Solanum commersonii (a wild potato) and PFG from petunia (Petunia hybrida) with 97% and 91% similarity over the entire protein length, respectively (Hart and Hannapel, 2002). Having a widespread expression pattern, POTM1 mRNA was detected in actively growing tissues like meristems, roots, new leaves, and new tubers (Kang and Hannapel, 1995). Like PFG, POTM1 transcripts are localized in tunica and corpus cells of vegetative and inflorescence meristems, leaf primordia, and the procambium (Immink et al., 1999; Hart and Hannapel, 2002). Despite the high sequence match and similarities in expression patterns, the role of POTM1 in development appears to be different from the role of PFG and other SQUA-like family members. Using a transgenic antisense approach, our results indicate that POTM1 regulates the balance of growth between axillary and apical vegetative meristems.

RESULTS

Suppression of POTM1 in Antisense Plants

POTM1 was isolated from an early-stage tuber cDNA library (Kang and Hannapel, 1995) that was constructed from 4-d-old axillary buds induced to form tubers. At this stage, tuberization is initiated and cells are undergoing rapid division and enlargement. Forty clones forming two groups were isolated from the library by using a probe containing only the conserved MADS box sequence. Further study indicated that the two groups most likely represent different alleles of POTM1, because they were 99% identical at the nucleotide level and 100% identical at the deduced amino acid level (Kang and Hannapel, 1995). Southern analysis indicated that POTM1 belongs to a small gene family and is present in a low copy number (Kang and Hannapel, 1996). Probes with and without the conserved MADS box sequence recognized only select genomic bands representative of a small gene family. On the basis of the results from the library screen and the Southern data, it is highly probable that our transgenic antisense strategy is suppressing only the accumulation of RNA from members of the POTM1 family of MADS box genes.

To elucidate the function of this regulatory protein, transgenic plants with suppressed levels of POTM1 mRNA expression were generated. The POTM1 cDNA sequence in the antisense orientation was placed under the control of the cauliflower mosaic virus 35S promoter to drive antisense transgene expression in all organs of the potato. The potato cv FL-1607 was transformed by the Agrobacterium tumefaciens (strain GV2260)-mediated leaf-disc transformation method (Liu et al., 1995). Transgenic plants were screened by PCR for the presence of the transgene by using primers specific for the kanamycin marker gene (nptII). A total of 34 independent transgenic lines that were nptII positive were assayed for antisense POTM1 transcript accumulation. Accumulation of the POTM1 antisense mRNA was not detected in two of the 34 nptII-positive transgenic lines. Of the 32 lines exhibiting POTM1 antisense expression, four independent transgenic lines with the greatest levels of antisense transcript accumulation were chosen for further analysis. Tissue culture plants that were transformed with the nptII gene alone were used as wild-type controls (WT). Sense 32P-labeled RNA probes were used to examine expression levels of the POTM1 antisense transgene in shoot tips of WT and independent suppression lines 2, 10, 15, and 23 (Fig. 1A). Whereas independent transgenic lines exhibited high levels of antisense transcript accumulation, as expected, expression of the transgene was not detected in RNA from WT plants. Accumulation of endogenous levels of POTM1 mRNA were examined with gene-specific antisense 32P-labeled RNA probes. Accumulation of POTM1 mRNA in all four suppression lines was reduced substantially compared with WT levels (Fig. 1B). Suppression line 23 exhibited the greatest reduction in native POTM1 mRNA. Visualization of ethidium bromide-stained ribosomal RNA was used to ascertain loading and transfer (Fig. 1C).

Phenotype of POTM1 Suppression Lines

Suppression of POTM1 resulted in plants with a disrupted pattern of growth in organs both above and below ground. Transgenic lines with suppressed levels of endogenous POTM1 mRNA exhibited a re-
bands were used to ascertain loading of lanes.

Figure 1. Accumulation of \( \text{POTM1} \) antisense and endogenous transcripts in suppression lines of \( \text{POTM1} \). Twenty-five micrograms of total RNA from shoot tips of WT and independent transgenic lines 2, 10, 15, and 23 were loaded per lane. \( ^{32} \text{P} \)-labeled RNA probes with the MADS box deleted were used in the hybridizations. A, \( \text{POTM1} \) sense RNA probes were used to examine the accumulation of antisense \( \text{POTM1} \). B, \( \text{POTM1} \) antisense RNA probes were used to detect endogenous \( \text{POTM1} \) mRNA. C, Ethidium bromide-stained rRNA bands were used to ascertain loading of lanes.

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production in plant height and leaf size (Fig. 2, A–C). Compound leaves of the most extreme phenotypes (lines 10, 15, and 23) had fewer leaflets and were less dissected than WT leaves (Fig. 2A). Suppression lines exhibited a 2- to 3-fold reduction in the petiole length compared with WT (Fig. 2B). Although the length of the internodes in the apical and middle regions of the plant was reduced, the greatest reduction in internode length was observed in more basal internodes. Suppression line 23 exhibited a 2.6-fold reduction in internode length compared with WT (data not shown). This reduction in internode length and a concomitant loss in apical dominance resulted in plants that were dwarf and compact (Fig. 2C). Suppression lines exhibited shoot clusters growing from the axillary buds of stems (Fig. 2D). Growth of these axillary buds was prolific, but limited, forming a dense cluster of truncated shoots and stolons as well as small tubers. Growth from axillary buds occurred in an acropetal fashion, with the oldest axillary buds at the base of the plant exhibiting increased growth first (data not shown). Both initiation and growth of roots was inhibited in suppression lines (Fig. 2E). Whereas 100% of the cuttings from WT plants had initiated roots after 17 d, initiation of roots was inhibited in suppression lines, with 88%, 63%, 11%, and 0% of the cuttings forming roots for suppression lines 2, 10, 15, and 23, respectively. Of the roots that did develop in suppression lines, there was an 18% to 98% reduction in the total number of roots formed and a 57% to 100% reduction in the percentage that formed lateral roots compared with roots from WT cuttings (data not shown).

A model petiole-leaf cutting system was used to examine the tuberization potential of suppression lines. This system is advantageous because it provides a reliable, synchronous, and uniform model for tuber development. It has been used in numerous studies examining discrete stages in tuber formation (Wheeler et al., 1988; Hannapel, 1991; Vreugdenhil et al., 1999). In WT plants, axillary buds of petiole cuttings from short-day-induced and long-day-noninduced plants will grow out as tubers and shoots, respectively (Fig. 3A; Hannapel, 1991). \( \text{POTM1} \) mRNA accumulates in total RNA from axillary buds of this system (Kang and Hannapel, 1996). To determine the specific location of \( \text{POTM1} \) mRNA accumulation, in situ hybridization in 4-d-old tuberizing axillary buds was performed. Accumulation of \( \text{POTM1} \) mRNA is visualized as an orange stain under dark-field microscopy. There was no staining observed in negative control sections probed with a \( \text{POTM1} \) sense riboprobe (Fig. 3B). \( \text{POTM1} \) transcripts were localized to both corpus and tunica cells of the shoot apical and axillary meristems. In the primary apical meristem, \( \text{POTM1} \) mRNA was detected primarily in the central and peripheral zones. In the axillary meristems, but not the primary apical meristem, \( \text{POTM1} \) transcripts were also present in the rib zone of the meristem. Accumulation of \( \text{POTM1} \) mRNA was also abundant in the procambium of young leaves and the developing stem and in the adaxial cells of young leaves (Fig. 3C).

In the present study, cuttings were taken from plants grown under short days for at least 2 weeks, inductive conditions for tuber formation. As expected, axillary buds from WT plants developed into tubers (Fig. 3D, left). In suppression lines, however, there was a proliferation of nonelongating shoots (Fig. 3, D [right] and E; Table I). Whereas only one cutting of a total of 70 produced a tuber in the four suppression lines, all 11 of the WT axillary buds examined produced tubers after 10 d (Table I). Under greenhouse conditions, WT plants produced an average tuber yield of 266 g plant\(^{-1}\), whereas yields from suppression lines exhibited a reduction ranging from 79% to 97% (Table I). The tuber yield of suppression line 15 was reduced by more than 30-fold.

Whereas growth was observed in only one meristem in WT axillary buds, in the suppression lines, several meristems were activated (Fig. 3, D and E). To examine the number and placement of additional meristems activated in the suppression lines, scanning electron microscopy was performed on axillary buds from petiole-leaf cuttings. In WT potato, one axillary meristem will develop in the junction of the stem and the adaxial base of the leaf. Axillary meristems are initiated early in development and are derived directly from the SAM. During development in the petiole-leaf cutting system, the primary axillary meristem will develop into a single tuber under
short days (Fig. 3A; Hannapel, 1991). A 5-d-old axillary bud from line 15 is shown in Figure 4A. The positions of the incipient leaf (P0) to the oldest leaf primordia (P7) are marked. One meristem (black arrow) is visible at the base of the adaxial side of the P7 leaf. In addition, another meristem (white arrow) is just emerging at the base of the P6 leaf. In an axillary bud from petiole cuttings of a WT plant, no meristems were observed in the base of the P6 or P7 leaf even after 9 d of growth (Fig. 4B, arrows). Figure 4C is a montage of two micrographs of the same magnification showing an 8-d-old axillary bud from line 15. A single meristem is observed at the bases of the P7 to P10 leaves (arrows). The development of the meristem at the base of the P10 leaf is advanced with six leaf primordia already present (Fig. 4C, right). In addition, in the meristem subtending the P10 leaf, an axillary meristem is just emerging at the base of the P6 leaf primordia (arrowhead). Whereas both the placement and number of axillary meristems is normal, the timing of development is precocious in the suppression line.

To examine the morphology of the increased growth observed in axillary buds of the petiole cuttings, light microscopy was performed. Longitudinal sections from suppression line 15 and WT were stained with toluidine blue to examine the histology of 5-d-old axillary buds. At this stage of development, WT axillary buds are just initiating radial growth associated with tuber formation (Vreugdenhil et al., 1999). In line 15, the cells of the meristem (Fig. 5A) are more condensed compared with WT.
(Fig. 5B), particularly in the rib zone (arrow) of the meristem and in young leaves. Enlargements of the meristems are shown in Figure 5, C and D. Compared with WT, active cell division (arrows) is observed in line 15. Amyloplasts (arrows) are present in the cells of both line 15 (Fig. 5E) and WT (Fig. 5F), however, there is an increased number of amyloplasts present in line 15. On the basis of this observation, the Periodic Acid-Schiff’s (PAS) reaction was performed on serial sections to specifically stain for non-soluble polysaccharides, including starch and cellulose (Fig. 5, G and H). A higher magnification of the boxed areas in Figure 5, G and H, is shown in Figure 5, I and J. There is an increase in the number of starch grains present in suppression line 15 (Fig. 5I, arrow), particularly in the cells of the leaf. In contrast to the elongated cells present on the abaxial side of the WT leaf (Fig. 5J, arrowhead), the abaxial leaf cells of line 15 are more irregularly shaped and less elongated (Fig. 5I, arrowheads). This decrease in cell elongation in newly formed leaves may explain the reduction in leaf size characteristic of the suppression lines. Plates in Figure 5 are representative micrographs of numerous sections that were examined.

An increase in the number of starch grains was observed in light microscopy sections of suppression line 15. To further examine starch content, the fourth and fifth expanded leaves from whole plants of WT and suppression lines were harvested, and starch was quantitated. The leaves of POTM1 suppression lines had 2- to 5-fold more starch than leaves of WT plants (Fig. 6). Line 15 had the greatest increase in starch with 13.1 mg starch 50 mg \(-1\) dry weight compared with 2.6 mg starch 50 mg \(-1\) dry weight for WT leaves.

The phenotype of the POTM1 suppression lines indicates that these plants may have altered cytokinin levels. To test this possibility, cytokinin analysis was performed on axillary buds of suppression line 15 and control plants by using HPLC. Both zeatin and isopentenyl types of cytokinins increased 2- to 3-fold in axillary buds from plants grown under both long-day and short-day conditions (Table II). The short-day axillary buds analyzed for cytokinins (buds taken directly from stock plants grown under short-day conditions) in Table II were from the same material that produced the axillary bud phenotypes shown in Figures 3, D and E, 4, and 5.

**DISCUSSION**

**POTM1 Suppression Activates Axillary Meristem Growth**

By using a transgenic approach, we demonstrate that suppression of POTM1 mRNA accumulation produces a phenotype exhibiting reduced apical

![Figure 4.](image_url)
dominance, increased lateral growth, induced formation of shoot clusters on the stem, increased starch accumulation in new leaves, and a reduction in tuber formation. These changes are accompanied by increases in cytokinin content and in activation of axillary meristem growth. There are two stages of axillary meristem development, the initial formation of axillary meristems and the subsequent growth of the meristem (Shimizu-Sato and Mori, 2001). Axillary meristem mutants usually affect one or both of these stages of development. The origin of axillary meristems differs between different plant species. In potato and tomato, axillary meristems are derived from meristematic cells that detach from the shoot apical meristem (Sussex, 1955), whereas in Arabidopsis, axillary meristems are initiated in leaf axils from cells on the adaxial surface of the subtending leaf (Schmitz and Theres, 1999). Growth of axillary meristems is usually inhibited by the shoot apical meristem, in a process called apical dominance (Shimizu-Sato and Mori, 2001). Auxin restricts, whereas cytokinin promotes, the growth of axillary meristems (Shimizu-Sato and Mori, 2001).

The most notable phenotype caused by the suppression of POTM1 is the increase in axillary meristem growth. This is most apparent in the compact...
growth habit of the plant and in the proliferation of shoot growth on the stems of whole plants and from the axillary buds of petiole cuttings that were induced to form tubers. Scanning electron microscopy (Fig. 4) indicated that initiation of meristem formation does not appear to be affected by suppression of POTM1 because both the number and placement of axillary meristems is normal. In both WT and POTM1 suppression lines, one axillary meristem will develop in the junction of the stem and the adaxial base of the leaf. There are several examples of other genes that regulate lateral branching in a similar way. The MAX genes of Arabidopsis repress shoot lateral branching (Stirnberg et al., 2002). MAX1 and MAX2 appear to specifically control axillary growth rate after axillary meristem initiation. The arx1 mutant of Arabidopsis has a decreased sensitivity to auxin and exhibits a highly branched shoot phenotype (Stirnberg et al., 1999). Loss of ARX1 function does not affect the formation of axillary buds but rather increases the rate of development of lateral shoots. Interestingly, both MAX2 and ARX1 may be involved in the ubiquitin-mediated degradation of regulatory proteins. In petunia, the recessive dad1-1 allele conditions a highly branched phenotype accompanied by a reduction in apical dominance and a decrease in internode length (Napoli, 1996). The nonallelic ramosus mutations of pea (Pisum sativum) cause the release of vegetative axillary buds leading to extensive lateral growth and a reduction in apical dominance (Beveridge et al., 1996). Grafting experiments between WT and dad mutants or between WT and rms mutants indicate that axillary bud growth is affected by an unidentified substance that is transported from the roots (Napoli, 1996; Beveridge et al., 2000). The results of these studies suggest a complex interaction among hormones and other signaling compounds that regulates apical dominance and lateral branch-
from dormancy (for review, see Cline, 1991; McKenzie et al., 1998; van der Graaff et al., 2001). Chatfield et al. (2000) demonstrated that basipetal auxin transport was a potent inhibitor of axillary meristem growth. Basal application of cytokinins, however, was able to overcome the effect of apically applied auxins, thereby promoting the growth of axillary meristems. The lateral suppressor mutant from tomato prevents the initiation of axillary meristem formation resulting in empty leaf axils (Schumacher et al., 1999). Expressing the A. tumefaciens ipt gene in lateral suppressor mutants increased cytokinin levels and produced typical cytokinin overproduction phenotypes, but did not rescue the empty leaf axil phenotype (Groot et al., 1995). In tomato, at least, cytokinin levels affect the growth of axillary meristems but not their initiation. In a similar fashion, the suppression of POTM1 mRNA accumulation in potato affects the growth of axillary meristems but not their initiation or location.

Creation of Multiple Sinks in POTM1 Suppression Lines

In addition to releasing axillary meristems from dormancy, cytokinins have a profound effect on the mobilization of nutrients. Li et al. (1992) showed that cytokinin activity can create a nutrient sink and that both 14C-labeled Suc and amino acids are mobilized to localized sites of high cytokinin accumulation. Amyloplast development and the increased transcription of starch biosynthesis enzymes are specifically induced by cytokinins in cultured tobacco cells (Miyazawa et al., 1999). Local synthesis of cytokinins in axillary buds of transgenic tobacco resulted in an increase in starch accumulation in the lateral shoots that formed (Guivarc’h et al., 2002). POTM1 suppression resulted in an increase in starch accumulation and active cell division in specific cells of meristems and leaves (Figs. 5 and 6). These events may be controlled by the localized activity of POTM1 to limit cytokinin accumulation to specific regions of the meristem.

In suppression lines, development of normally strong vegetative sinks like the SAM and underground tubers are superceded by the induced secondary growth of axillary meristems. Whereas the axillary bud from WT cuttings of a model tuber system produces a single tuber, axillary buds from POTM1 suppression lines produce a proliferation of shoots. These results support the premise that the relationship between the growth of lateral organs and the apical meristem are antagonistic. In Arabidopsis, recessive mutants of the revoluta gene (Talbert et al., 1995) are characterized by longer, thicker leaves and decreased meristem formation. Growth of lateral organs like leaves is favored over growth of meristems, often leading to an arrest in apical meristem development. It is postulated that the function of REVOLUTA is to promote apical meristem growth and to limit cell division and elongation in leaves and stems (Talbert et al., 1995). In contrast, POTM1 suppression lines exhibited smaller leaves and more active lateral meristem growth. POTM1 suppression produced an increase in cell division in meristems and created multiple new sinks at the expense of leaf expansion and tuber formation. An extreme example of this was demonstrated by the increase in shoot/stolon/tuber growth arising from axillary stem buds in select suppression lines accompanied by a concomitant reduction in underground tuber yields.

The phenotypes of the suppression lines, particularly activated axillary meristem growth, increased cell division, decreased tuber yields, and selective starch accumulation, suggest that POTM1 may be involved in regulating the balance of growth in vegetative meristems, favoring the development of a dominant sink organ. The increased levels of cytokinins in axillary buds of suppression line 15 support this premise. For the aboveground portion of the plant, this sink organ is the SAM. In stolon/tuber development, the sinks are a few enlarging tubers that form underground. In the model petiole-leaf cutting tuberization system, the single tuber produced is a very strong sink. Suppression of POTM1 may alter the ratio of auxins and cytokinins (by increasing the level of cytokinins, for example) to activate growth of lateral meristems at the expense of the apical or dominant shoot organ (SAM or tuber). It is conceivable that reduction in POTM1 mRNA expression enhances cytokinin activity in specific cells of the vegetative meristem leading to increased cell division in meristems and a disruption in WT source/sink interactions.

MATERIALS AND METHODS

Transformation of POTM1 Antisense Plants

The full-length POTM1 cDNA (Kang and Hannapel, 1995) in the antisense orientation was cloned into the binary vector, pCB201 (Xiang et al., 1999), under the control of the cauliflower mosaic virus 35S promoter. This plasmid was transformed into Agrobacterium tumefaciens strain GV2260. The leaf-disc transformation method (Liu et al., 1995) was used to transform the construct into potato (Solanum tuberosum cv FL-1607). The presence of the transgene was confirmed by PCR primers specific for the kanamycin-marker gene (nptII; data not shown). A total of 34 independent transgenic lines that were nptII positive were screened for the accumulation of POTM1 antisense mRNA. Four independent transgenic lines that exhibited high levels of POTM1 antisense mRNA accumulation were selected for further analysis. Tissue culture plants transformed with the nptII marker gene alone were used as controls (WT). For subsequent experiments, all lines were propagated vegetatively from bud cuttings in vitro and transferred to soil after rooting.

RNA-Blot Hybridization

Total RNA was extracted from shoot tips of independent transgenic lines and controls with TriPure Isolation Reagent (Roche Diagnostics, Indianapolis). Using 10 mM methyl mercury (II) hydroxide as a denaturant, 25 μg of total RNA was subjected to gel electrophoresis and blot transfer. POTM1 with the conserved MADS box deleted was cloned into the pCRII vector (Invitrogen, Carlsbad, CA) flanked by the SP6 and T7 promoters. The vector

was linearized with XhoI or BamHI and transcribed with the SP6 or T7 RNA polymerase for sense and antisense probes, respectively. [a-32P]PCTP was incorporated into RNA probes according to the manufacture’s instructions (Promega, Madison, WI). The membrane was hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX) at 68°C for 16 h. Washing was as follows: 2× SSC and 0.1% (w/v) SDS at room temperature for 5 min; 1× SSC and 0.1% (w/v) SDS at 68°C for 20 min; and 0.1× SSC and 0.1% (w/v) SDS at 68°C for 20 min. The film was exposed for 4 d at −80°C.

Tuber Yields from Petiole-Leaf Cuttings and Whole Plants

For petiole-leaf cuttings, whole plants were induced under a short-day photoperiod (8 h of light/16 h of dark) for at least 2 weeks. Cuttings were cultured in a perlite-vermiculite mix with the axillary bud placed below the surface under a short-day photoperiod in a growth chamber (Hannapel, 1991). After 10 d, the axillary buds were examined for morphology, growth, and tuber formation. Growth was allowed to continue for at least 19 d before photographs in Figure 3 were taken on a S2H10 Research Stereo Microscope (Olympus, Melville, NY). For whole plants, tuber yields were measured from 100-d-old plants that were grown in the greenhouse under a long-day photoperiod (16 h of light/8 h of dark).

In Situ Hybridization

WT plants were grown under short-day conditions (8 h of light/16 h of dark) for at least 2 weeks. In situ hybridizations with gene-specific probes for POTM1 were performed on 4-d-old axillary buds from petiole-leaf cuttings cultured as described above. In situ hybridizations were performed as described previously (Hart and Hannapel, 2002).

Light and Scanning Electron Microscopy

WT and independent suppression line 15 plants were grown under short-day conditions (8 h of light/16 h of dark) for at least 2 weeks. Petiole-leaf cuttings were cultured as described above, and meristems were harvested daily and fixed in 2% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde in either 0.1 m cacodylate buffer, pH 7.2, or 0.1 m sodium phosphate buffer, pH 7.0, at 4°C for 2 to 11 d. After fixation, the tissue was dehydrated in a graded ethanol series. Tissue for light microscopy was embedded in LR White (Electron Microscopy Sciences, Ft. Washington, PA), and 1.4-μm sections were cut on an ultramicrotome (Reichert/Leica, Deerfield, IL). For general morphology, sections were stained with 1% (w/v) toluidine blue. The PAS reaction was performed on serial sections to stain for non-water-soluble polysaccharides. Negative controls for the PAS reaction, in which one reagent was missing (periodic acid), did not show any staining (data not shown). Bright-field microscopy was used to photodocument sections. Tissue for scanning electron microscopy was critical point dried with six flushes of CO2, mounted on aluminum stubs with silver paint, and coated with gold/palladium. Specimens were examined on a scanning electron microscope (JSM-5800LV, JEOL, Tokyo) at the Bessey Microscopy Facility, Iowa State University, for continuing support and expertise in microscopy techniques.

Starch Assay

The fourth and fifth expanded leaves from whole plants of WT and independent suppression lines were harvested in liquid N2 and lyophilized. Samples were extracted overnight in Bieleski extraction solution (24,000 g, 15 min, 4°C) deuterated standards for cytokinins (Apex International, Honiton, UK) were added. The extract was purified using a combination of solid phase and immunoaffinity purification as described by Redig et al. (1996). Quantitative analysis of cytokinins was performed by means of capillary column switching on a fully automated Famos workstation (LC Packings, Amsterdam) coupled to a liquid chromatograph set-up consisting of a 3255 HPLC pump (Kontron Instruments, Milan), an in-line Kontron 322 UV detector, and a triple quadrupole mass spectrometer (Quattro II, Micromass UK Ltd, Cheshire, UK). Twenty-five-microliter sample aliquots were introduced into one dimension of the system using 10 mm CH3COONH4 as the mobile phase at a 40 μL min−1 flow rate. During 7 min, analytes were captured on a micromer precolumn (C18, 5 μm, 500 μm i.d. × 5 mm; LC Packings) followed by a mobile phase switch for 5 min using 10 mm CH3COONH4, water:methanol (30:70, v/v) at a 7 μL min−1 flow rate as mobile phase to introduce the analytes onto the analytical column (Adsorbosphere C18, 5 μm, 300 μm i.d. × 150 mm, Alltech, Laarne, Belgium). Cytokinin chromatograms were recorded in multiple reaction-monitoring mode using diagnostic transition ions as described by Prinsen et al. (1995). Absolute detection limits ranged from 2 fmol for isopentenyl to 15 fmol for zeatin. Results were calculated according to the principle of isotope dilution and expressed in picomoles per gram fresh weight.

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