Meristem activity during flower and ovule development in tomato is controlled by the mini zinc finger gene INHIBITOR OF MERISTEM ACTIVITY

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Summary

Plants have the ability to form new organs as a result of indeterminate growth ensured by specific regions of pluripotent cells, called meristems. Flowers are produced by the activity of floral meristems which differ from vegetative meristems in their determinate fate. Transcriptional complexes associating C-, D- and E-type MADS box proteins are responsible for flower determinacy by controlling the stem cell population within the floral meristem. We report here that the INHIBITOR OF MERISTEM ACTIVITY (IMA) gene encoding a mini zinc finger (MIF) protein from tomato (Solanum lycopersicum) regulates the processes of flower and ovule development. IMA inhibits cell proliferation during floral termination, controls the number of carpels during floral development and acts as a repressor of the meristem organizing centre gene WUSCHEL. Underexpression of IMA prevents the formation of gametophytic tissue, which is replaced by an undetermined sporophytic tissue. Molecular analyses of MADS box gene expression revealed that IMA participates in the termination of floral meristems and initiation of ovule primordia by activating D-type gene expression. Taken together, our data indicate that both termination of floral meristems and differentiation of nucelli during ovule development require a similar mechanism involving the repression of WUSCHEL and the activation of D-class genes.

Keywords: floral meristem, MIF protein, ovule development, carpel, tomato.

Introduction

Development and growth of vascular plants is continuous throughout the plant life cycle. As a result vascular plants have the ability to form new organs due to indeterminate growth which is ensured by specific regions of pluripotent cells, called meristems. The shoot apical meristem (SAM) consists of a central zone (CZ) comprising an organizing centre (OC), which underlies the stem cell niche, surrounded by peripheral zones (PZ). Our understanding of how the SAM functions has been enriched by many recent advances using genetic dissection of Arabidopsis mutants (for a review see Castellano and Sablowski, 2005). The fate of stem cells in the CZ is specified by the homeodomain protein WUSCHEL (WUS) which is specifically expressed in the OC of the CZ, and acts through a non-cell-autonomous mechanism (Laux et al., 1996; Mayer et al., 1998). During floral induction, the SAM is converted into an inflorescence meristem which in turn gives rise to several floral meristems (FM; for a review see Blazquez et al., 2006). Two major genes LEAFY (LFY) and APETALA1 (AP1) specify the identity of the FM. Unlike the SAM, the FM displays determinate growth leading to the formation of a limited number of organs before the cessation of meristematic activity. This process, called floral termination, is under the control of a negative feedback loop involving the meristematic organizer gene WUS and the AGAMOUS (AG) gene (Lenhard et al., 2001; Lohmann et al., 2001). After floral induction LFY and WUS are expressed and activate AG in the centre of the FM which in turn represses WUS to arrest floral development.
A set of genes specifies floral organ identity according to four distinct functions, called A, B, C and E (for a review see Jack, 2004). Carpel identity is under the control of C- and E-type genes in the fourth whorl (Castillejo et al., 2005; Ferrario et al., 2008; Robles and Pelaz, 2005). Ovule identity in the centre of the carpel is under the control of a fifth class of genes named D-type genes (Colombo et al., 1995) that acts in combination with C- and E-type gene activities (Favaro et al., 2003; Ferrario et al., 2006).

The development of the ovule proceeds in four distinct phases (Schneitz et al., 1997). The first phase corresponds to the initiation of the ovule primordia from the placenta inside the carpel. The specification of ovule identity occurs during the second phase, immediately followed by the formation of spatially defined patterns within the developing ovule in the third phase. During the final fourth phase, the distal portions of ovule primordia develop in the nucellus containing the megaspore mother cell (MMC). The MMC then undergoes meiosis to produce four megaspores of which only the top one near the micropyle survives to ultimately form the embryo sac. In angiosperms, ovules consist of a nucellus enclosed by one or two integuments and a supporting funiculus which links the ovule to the placenta (Bouman and Meijer, 1994; Reiser and Fischer, 1993). Ovule development is under tight genetic control (Skinner et al., 2004), and since ovules derive from the placenta as specific meristematic regions, regulatory genes such as WUS play a key role in ovule organogenesis (Gallois et al., 2002). Indeed, WUS is expressed in ovule primordia and contributes to the initiation of the formation of the female gametophyte and the generation of the integuments during ovule development (Gross-Hardt et al., 2002; Skinner et al., 2004). Restriction of WUS expression takes place ultimately during ovule termination.

Recently, a new family of proteins harbouring a putative zinc finger of the CX3HX11CX12-26CX2CXCHX3H type (Windhövel et al., 2000) has been characterized in Arabidopsis (Hu and Ma, 2006). Due to their very small size (~100 amino acids) these proteins have been named MINI ZINC FINGER (MIF) proteins. MIF1 as a representative member of this protein family was shown to be involved in multiple hormonal regulatory pathways during Arabidopsis development (Hu and Ma, 2006). Unfortunately, no loss-of-function mutants have been described so far, thus hampering elucidation of the function of MIF1. In this study, we describe a novel regulator of meristem activity in tomato (Solanum lycopersicum), called INHIBITOR OF MERISTEM ACTIVITY (IMA) and show that it encodes such a MIF protein. The in planta functional analysis of IMA described here shows that it acts as a repressor of the meristem OCE gene SIWUS and inhibits cell proliferation during floral termination. Moreover, IMA was shown to be involved in the initiation of ovule primordia and the determinacy of the ovule nucellus.

Results

IMA is a member of the mini zinc finger protein family

The 617-bp long IMA cDNA (accession no. AM261628) was identified in a differential hybridization screen aimed at isolating genes preferentially expressed at the cell division phase of early fruit development (Joubès et al., 1999). IMA encodes a 90 amino acid (aa) long protein, IMA (Mr = 9000), which belongs to the recently characterized MIF protein family (Hu and Ma, 2006), as it harbours a putative central and unusual zinc finger (ZF) domain (encompassing residues 27 to 72) primarily identified in Flaveria ZF-HD proteins (Windhövel et al., 2000). So far, in tomato, only two members have been identified: IMA which shares the highest percentage of identity with the Arabidopsis MIF2 protein (62%), and a MIF1-related protein (SIMIF1-like) displaying 57% identity with both Arabidopsis MIF1 and MIF3.

IMA is preferentially expressed during reproductive development in tomato

While absent from vegetative organs, IMA transcripts could be detected during the time course of fruit development as early as the pre-anthesis (PA) stage corresponding to isolated carpels (Figure 1a). Then IMA transcripts accumulated gradually and strongly in fruit to reach a maximum at 10 days post-anthesis (dpa), and then decreased to reach a basal level at the mature green (MG) stage corresponding to growth termination and the onset of ripening.

During floral development, IMA expression was not detected in stage 1 flower buds (for a definition of developmental stages see Brukhin et al., 2003) as shown by in situ hybridization (Figure 1b). IMA started to be expressed in the CZ, as well as in the petal primordia of the FM at stage 2. Later on, and during the process of floral termination (stage 6), the IMA transcripts accumulated in the centre of the carpel primordium and the apical parts of growing sepals and petals. Using RT-PCR analysis, we demonstrated that the expression of IMA in mature flowers is detected in all floral parts, but to a greater extent in carpels and petals (see Figure S1).

Inside the carpel, IMA transcripts accumulated within the placenta during initiation of the ovule primordium (Figure 1c), and later on in every cell of the developing ovule primordia. IMA was strongly expressed in the inner integuments as the nucellus became enclosed, while very slight mRNA expression was observed in the nucellus. Later on the IMA transcripts were barely detectable in integuments of mature ovule while no signal was associated with the embryo sac (megagametophyte).
The ectopic overexpression of IMA affects plant development

Fifteen tomato transgenic lines overexpressing the complete IMA coding sequence under the control of the cauliflower mosaic virus (CaMV) 3S promoter (referred to as Pro35S:IMA) were generated. Among the whole set of IMA overexpressors no line was found with IMA co-suppression. Four independent lines, referred to as Pro35S:IMA1 to Pro35S:IMA4, overexpressing IMA to various extents, were then selected (see Figure S2). Surprisingly, the maximum IMA overexpression ever measured (found in the Pro35S:IMA4 line) did not go beyond a two-fold increase in IMA transcript abundance when compared with wild-type (WT) plants, suggesting a potential drastic effect of the transgene on plant development.

In the different overexpressor lines, the gradation in the level of expression of IMA correlated with the observed phenotypes (Figure 2 and Figure S2). Pro35S:IMA1 plants were indistinguishable from WT plants, while a gradual reduction in plant height was observed from the Pro35S:IMA2 to the Pro35S:IMA4 line (Figure 2a). This reduction in plant size was likely to be due to the slower growth rate associated with a loss of apical dominance, resulting in a bushy appearance (Figure 2b). In addition to size alteration, leaf morphology and colour were also affected: leaves were abnormally curved showing increased epinasty with a dark green colour (Figure 2c). All these phenotypes were quite similar to those obtained with the ectopic overexpression of MIF1 in Arabidopsis (Hu and Ma, 2006), and interestingly we could also demonstrate that the overexpression of IMA also induces a reduced sensitivity to the effects
Figure 2. Vegetative phenotypic analysis of *IMA* overexpressor transgenic lines.
(a) Growth alteration of 4-week-old *IMA* overexpressor transgenic plants compared with wild type (WT).
(b) Comparison of whole 3-month-old plant phenotypic analysis of *Pro35S:IMA4* with WT.
(c) Leaf shape phenotype in *IMA* overexpressor transgenic lines.
(d) Hormonal responses in *Pro35S:IMA2* and *Pro35S:IMA4* to increasing concentrations of auxin (IAA) and cytokinin (6-BAP). Hormonal responses were assessed by measuring root length. Data are expressed as relative root length reported to the length of untreated WT roots. Typical seedling developments are represented for the *Pro35S:IMA4* line.
of both auxin (IAA) and cytokinin (6-BAP) on root elongation, as illustrated for Pro35S:IMA2 and Pro35S:IMA4 seedlings (Figure 2d).

The Pro35S:IMA2 to Pro35S:IMA4 plants produced smaller flowers and fruits (Figure 3a). As shown for Pro35S:IMA4 plants, these smaller flowers harboured a reduced number of seeds, and pollination using WT pollen significantly increased the number of seeds per fruit (Figure S2a). This deficiency in Pro35S:IMA4 pollen grains was further confirmed by pollen germination tests, clearly showing an alteration in pollen tube elongation (inset of Figure S3a). Although dwarf, Pro35S:IMA flowers contained a larger ovary than WT flowers (Figure 3b). These ovaries were full of normal-shaped ovules (Figure 3c) and displayed a significantly increased number of ovules (up to 1.25-fold more in the Pro35S:IMA4 line than in WT).

Despite the reduction in seed number and germination rate observed in the Pro35S:IMA lineage, a T1 progeny was obtained in which the T0 phenotypes were reproduced. In the Pro35S:IMA2 and Pro35S:IMA4 T1 progeny, the development of the hypocotyl and primary root of 10-day-old seedlings was also strongly affected (see Figure S3), suggesting that the reduction in plant size caused by IMA overexpression is not exclusively due to a loss of apical dominance but also to an alteration in plant growth. At the cellular level the overexpression of IMA induced a spectacular decrease in cell size as measured for stem epidermis cells (Figure S3). This reduction in cell size was also observed whatever the source of the organ, suggesting that it was responsible for the alteration of plant growth and organ size in IMA overexpressing lines.

IMA controls the number of carpels in tomato fruit

Ten transgenic lines harbouring an IMA antisense RNA construct (referred to as Pro35S:ima×1) and 11 transgenic tomato lines harbouring a RNA interference (RNAi) construct (referred to as Pro35S:ima) were generated to decrease the endogenous expression of IMA in tomato. Resulting from the loss-of-function strategies, the Pro35S:ima×1, Pro35S:ima×2, Pro35S:ima1 and Pro35S:ima2 lines were selected as they exhibited a significant reduction in IMA transcript abundance (see Figure S2).

No particular phenotypic alterations in vegetative development were observed for the antisense and RNAi plants. However, these lines displayed an enlarged ovary. The accelerated enlargement of the ovary resulted in the stigma being positioned out of reach of the stamens, impairing self-pollination and contributing to the development of seedless fruit in the worst cases. Since the Pro35S:ima×1, Pro35S:ima×2, Pro35S:ima1 and Pro35S:ima2 lines behave identically, the Pro35S:ima1 line will be presented hereafter as the IMA loss-of-function reference line (Figure 4). When compared with the ovaries of WT flowers which result from the fusion of two carpels, the fourth whorl of Pro35S:ima1 flowers gave rise to three to nine ovaries (Figure 4a). These supernumerary ovaries arose from the centre of an enlarged FM (Figure 5b, top right panel), thus suggesting that the meristem displays an enhanced activity. In about 20% of Pro35S:ima1 flowers this alteration initiated multiple ovary primordia which finally gave rise to multiple independent ovaries instead of a single one (Figure 4b, bottom right panel). This alteration in reproductive development was inherited in accordance to a 3:1 ratio in the T1 progeny of the transgenic RNAi lines.

In contrast to the WT ovary containing round ovules, the ovaries of loss-of-function lines as illustrated for Pro35S:ima1 plants enclosed finger-shaped ovules.
(Figure 4c), thus suggesting an overgrowth of the integument or an indeterminacy of the ovule structure during development. A clear correlation was observed between the number of modified ovules and the reduction in \textit{IMA} expression in the different transgenic loss-of-function tomato lines (Table 1), thus highlighting the penetrance of such striking phenotypic alterations. In the \textit{IMA} loss-of-function plants, ovule development was indistinguishable from that of the WT in the very early developmental stage corresponding to the emergence of the ovule primordia from the placenta until the formation of the MMC. However, we could not observe any MMC formation in Pro\textit{35S:ima1} ovules (Figure 4d, top right panel) when compared with WT ovules (Figure 4d, top left panel). In addition we noted cell proliferation in Pro\textit{35S:ima1} carpels showing the characteristic finger-like structure; note the cell mass in the megagametophyte presumptive region.

**IMA negatively regulates cell division**

As shown in Figure 5(a), the carpel primordia within the FM (characterized by cell division activities) were strongly reduced in the Pro\textit{35S:IMA4} overexpressor. Conversely they were spectacularly enlarged in the transgenic RNAi Pro\textit{35S:ima1} line when compared with the WT. The modifications in carpel size were likely to originate from an alteration in the cell number. Indeed, when compared with WT, cell layers were significantly less numerous in the carpel wall from the severely affected Pro\textit{35S:IMA4} line while the number of cell layers was greatly increased in the carpel wall of the Pro\textit{35S:ima1} line (Figure 5b), thus suggesting that \textit{IMA} encodes an inhibitor of cell division. The expression of cell cycle-regulated genes was shown to display converse...
behaviours in Pro35S:IMA4 and Pro35S:ima1 mature carpels, and therefore supports this role for IMA in the inhibition of cell division (Figure S4).

IMA contributes to termination of the floral meristem by inhibiting WUS expression

The formation of new carpels in the Pro35S:ima1 line may be linked with an impairment of arrest of meristem activity at the end of flower development, together with an increased availability in stem cells within the meristem. In Arabidopsis thaliana, WUSCHEL has been shown to play a key role in the maintenance of the stem cell niche and meristematic cell supply. Hence we analysed the spatial and temporal expression of the tomato WUSCHEL orthologue (Reinhardt et al., 2003; Stuurman et al., 2002), SIWUS, in floral meristems of WT, Pro35S:ima1 and Pro35S:IMA4 plants using in situ hybridization.

In developing flowers at stage 4, the SIWUS transcripts were still expressed in the OC of the FM in the Pro35S:ima1 transgenic RNAi lines, while the expression of SIWUS was no longer detected in the OC of the FM in WT plants, in accordance with its expected expression territories (Lenhard...
et al., 2001). Similarly the SlWUS transcripts were totally absent from the OC in the Pro35S:IMA4 line (Figure 5c). Thus IMA may be required for the extinction of SlWUS expression and to promote the proper arrest of FM activity.

To decipher the genetic pathway by which IMA may affect SlWUS expression, the expression of genes involved in the control of FM determinacy was also investigated (Figure 5c). The tomato orthologues for AGAMOUS (TAG1) (Pnueli et al., 1994), FB7/11 from petunia (TAG11) (Busi et al., 2003), SEPALLATA (TM29; accession no. AJ302015) and AINTEGUMENTA (SIANT; accession no. B1927245) were then used as respective marker genes for the C function, D function, E function and for maintenance of cell division activity (Klucher et al., 1996; Krizek, 1999). In WT the expression of TAG1 was located in the centre of the FM at stage 4 (Figure 5c), while it was excluded from the centre of the FM and restricted to the supernumerary presumptive carpel regions of the meristem in the Pro35S:ima1 line. No spatial modification of TAG1 expression could be observed in the Pro35S:IMA4 line when compared with WT. Interestingly, transcripts for the D-function gene TAgl11 were shown to accumulate strongly in the fourth whorl in the Pro35S:IMA4 line, and this expression extended to the stamen whorl when compared with WT. We could not detect any hybridization signal for TAgl11 in the Pro35S:ima1 line. The expression of the E-function gene TM29 was not significantly modified between WT and the Pro35S:ima1 and Pro35S:IMA4 plants (see Figure S5). As expected from its function in promoting cell division, SIANT was highly expressed in the central part of the floral meristem at stage 4 in the Pro35S:ima1 line (Figure 5c), in accordance with the observed primordial outgrowth (Figure 4b). However, we could not observe any significant difference in SIANT expression in the Pro35S:IMA4 line when compared with that of WT. We conclude from these data that the maintenance of SlWUS expression in the FM of Pro35S:ima1 line correlated with a modification of the expression territories of the C-function gene (TAG1) and the D-function gene (TAgl11), which in turn might affect the proper floral meristematic activity and lead to the formation of additional carpels.

IMA inhibits WUSCHEL expression and promotes ovule-specific marker gene expression during development of the nucellus

In Arabidopsis, WUSCHEL not only plays a central role in the initiation and maintenance of stem cell fate in the apical meristem, but also in ovule development (Gross-Hardt et al., 2002). At the stage when ovule primordia emerge from the placenta, a very slight expression of SlWUS was detected at the top of the ovule primordia in WT as well as in Pro35S:ima1 and Pro35S:IMA4 (Figures 6a–c). At the MMC forming stage, SlWUS was strongly upregulated in the nucellar cells surrounding the MMC in Pro35S:ima1 plants (Figure 6e), whereas the corresponding signal was absent in WT (Figure 6d) and Pro35S:IMA4 (Figure 6f) ovules. This suggests that IMA inhibits SlWUS expression during ovule development after initiation of the ovule primordium and at the MMC forming stage.

Since the modifications in IMA gene expression give rise to finger-shaped ovules and result in an increased number of ovules in loss-of-function and gain-of-function plants, respectively, the expression of TAgl11 as a D-function gene specifying ovule identity (Angenent et al., 1995) was investigated in Pro35S:ima1 and Pro35S:IMA4 plants. When compared with WT (Figure 6g), the level of TAgl11 transcripts
was severely reduced in Pro\textsubscript{35S}:ima\textsubscript{1} plants so as to be almost undetectable (Figure 6h). In contrast, the over-expression of IMA resulted in the activation of TAgl11, in accordance with the overproduction of ovule primordia (Figure 6i). Since \textit{AINTEGUMENTA} is known to control the development of integument in Arabidopsis through regulation of cell proliferation by modulating \textit{CYCD3} expression (Mizukami and Fischer, 2000), we also investigated the expression of \textit{SIANT}. As expected in WT ovules, \textit{SIANT} expression was detected in the developing integument but was absent from the nucellus (Figure 6j). In ovules of Pro\textsubscript{35S}:ima\textsubscript{1} plants \textit{SIANT} was upregulated in the developing integument together with a strong hybridization signal in the upper part of the nucellus (Figure 6k). At the mature stage, \textit{SIANT} expression was still detected in WT ovules (Figure 6m) but remained high in the integument of Pro\textsubscript{35S}:ima\textsubscript{1} ovules (Figure 6n) as expected for its function as a regulator of cell proliferation during development of the integument. Consistent with the normal development of the ovules in the Pro\textsubscript{35S}:IMA4 overexressor line, the \textit{SIANT} expression pattern was unaffected when compared with that of WT (Figure 6l,o).

**Discussion**

The \textit{INHIBITOR OF MERISTEM ACTIVITY (IMA)} gene belongs to the recently identified \textit{MINI ZINC FINGER (MIF)} gene family (Hu and Ma, 2006). While in Arabidopsis the expression patterns of \textit{MIF1} to \textit{MIF3} overlap, the two \textit{MIF} genes so far identified in tomato, \textit{IMA} and \textit{SlMIF1-like}, display a clear differential expression pattern restricted to reproductive (Figure 1a) and vegetative organs (Figure S1), respectively. Neither EMS nor insertional mutants for \textit{MIF} genes are available in Arabidopsis or tomato, which has so far hampered the functional analysis of \textit{MIF} genes. Moreover, Hu and Ma (2006) reported that complete inhibition of \textit{MIF} expression could result in lethality in Arabidopsis. The present detailed functional analysis of \textit{IMA} took advantage of the availability of transgenic loss-of-function plants for a \textit{MIF} gene.

\textit{IMA} regulates plant development in specific territories by limiting cell division

Maximum IMA expression during fruit development coincides with the transition between the cell division and cell expansion phase of growth, i.e. slightly before 10 dpa (Cheniclet \textit{et al.}, 2005; Gillaspy \textit{et al.}, 1993; Joubès \textit{et al.}, 1999). At this stage cell divisions cease, and thereafter fruit growth to almost full size is principally obtained through cell expansion. During flower development, the expression of \textit{IMA} was ultimately restricted to the central fourth whorl of the flower at a time when the FM activity normally decreases, and during FM termination (Figure 1b). This expression coincides spatially and temporally with the early development of ovules.

When ectopically overexpressed, IMA induces pleiotropic effects such as reduced plant growth, loss of apical dominance and curly leaves, thus indicating that IMA can be active and functional in territories other than those where it is normally expressed, i.e. flowers and fruits. The reduction in size of flowers and fruits in the IMA overexpressors is the result of a narrowing of the CZ in the FM and consequent impairment of cell division activity. Even though the reduction in IMA expression in the RNAi transgenic lines has no effect on the other developing floral organs, or on the vegetative growth of the whole plant, it clearly induces converse effects to those displayed by IMA overexpressors during flower development, i.e. an increased size of the FM which ultimately gives rise to the formation of supernumerary carpel primordia originating from the central fourth whorl. In accordance with the endogenous expression profile, the phenotypes induced by the alteration of IMA gene expression indicate that IMA acts as a general inhibitor of plant growth and development by impairing cell proliferation, and more specifically by participating in FM termination.

\textit{IMA participates in the control pathway of WUSCHEL during flower meristem termination}

\textit{WUS} is expressed in the CZ of the FM early during flower development to maintain the meristematic activity necessary for the formation of flower organs (Lenhard \textit{et al.}, 2001). Later on, as the flower develops, its expression rapidly decreases and becomes undetectable in the FM. We show here that the expressions of IMA and \textit{SlWUS} are mutually exclusive during flower development in tomato (Figures 5 and 6). Interestingly, the maintenance of \textit{SlWUS} expression correlates directly with the area with greatest potential to form new organs in the centre of Pro\textsubscript{35S}:ima\textsubscript{1} flowers (Figure 4), and thus with the maintenance of meristem activity which impedes FM termination.

In Arabidopsis and petunia, termination of the FM requires the expression of C-, D- and E-function genes. The impairment of C and E functions causes an upregulation of \textit{WUS} in the fourth whorl and the consequent loss of floral determinacy (Ampomah-Dwamena \textit{et al.}, 2002; Angenent \textit{et al.}, 1994, 1995; Dreni \textit{et al.}, 2007; Ferrario \textit{et al.}, 2003; Kapoor \textit{et al.}, 2002; Pnueli \textit{et al.}, 1994). Furthermore a recent study suggests a combinatory role for E- and D-function genes in the downregulation of the petunia \textit{WUS} orthologue (named \textit{TERMINATOR}) during floral termination (Ferrario \textit{et al.}, 2006). This repression of \textit{WUS} is thought to be mediated by transcription factor complexes consisting of C-, D- and E-function MADS box proteins, thus leading to floral determinacy specifying carpel and ovule identity (Ferrario \textit{et al.}, 2006).
At the transcriptional level, altering the expression of IMA has no significant effect on the expression of the C-function gene TAG1 (Figure 5c) and the E-function gene TM29 (a SEPALLATA orthologue in tomato; Ampomah-Dwamena et al., 2002; Figure S5). On the contrary, IMA induces the D-function gene Tagl11 (Figure 5c). Our data thus indicate that IMA controls flower determinacy by activating the D function, since supernumerary carpels arise in the centre of the FM of Pro35S:ima1 plants while D-type transcripts are strongly repressed. This was not observed in Arabidopsis as neither the stk mutant for the D-function gene nor the shp1 shp2 stk triple mutant displays indeterminate flowers (Pinyopich et al., 2003), indicating that the D function is not involved in flower determinacy. An explanation for this difference can rely on the specific pattern of development for ovules in the different species. In Arabidopsis the ovules develop from the merging of the carpel valve, while in species such as tomato, petunia and rice (Oryza sativa) the ovules develop directly from the centre of the FM. Interestingly the mutation of the D-type gene in rice, OsMADS13, leads to the formation of indeterminate flowers (Dreni et al., 2007) as observed for Pro35S:ima1 plants.

IMA controls differentiation of the nucellus

The upregulation of IMA increased the number of ovules as a result of its positive regulation on Tagl11 expression which specifies ovule identity (Ferrario et al., 2006). Conversely the downregulation of IMA in Pro35S:ima1 plants resulted in the repression of Tagl11 and a dramatic impairment in the differentiation of both nucellus and ovule, giving rise to undifferentiated finger-like structures primarily comprising integument tissue and devoid of embryo sac (Figure 4). These observations suggest that the genetic program inhibited by IMA remains active within the nucellus, thus preventing the production of the MMC. The maintenance of cell proliferation in the region normally occupied by the MMC is defined molecularly by sustained SIWUS expression in Pro35S:ima1 ovules (Figure 6e), according to its supposed function reported by Gross-Hardt et al. (2002) in development of both the integument and nucellus. In accordance with previous studies (Ampomah-Dwamena et al., 2002; Favaro et al., 2003; Gross-Hardt et al., 2002; Payne et al., 2004), our data therefore indicate that the shift from the indeterminate state to the determinate state and then to nucellus differentiation is intimately linked to the repression of meristem-associated genes during ovule development, as a result of inhibition of SIWUS within the nucellus primordium, under the direct or indirect control of IMA.

IMA controls cell proliferation and meristem activity

We have proposed a tentative model for the regulatory pathway required for FM termination and nucellus differentiation during ovule development (Figure S6). According to Ferrario et al. (2006), once activated in the centre of the FM or in the centre of the developing ovule, IMA would activate the D-function genes leading to the interaction of C, D and E proteins and consequently to the repression of the gene regulating stem cell fate, SIWUS. As a result, inhibition of meristem activity and the promotion of ovule identity would occur. The recent work of Brambilla et al. (2007) argues for the repression of WUS via the activation of D-function genes during ovule development in Arabidopsis, since the BEL1–AG–SEP complex repressing WUS is stabilized by the STK–SHP–SEP ovule identity complex.

Plant hormones exert a tight control on cell division, the meristem structure, the meristem dormancy or activity and the resulting formation of organ primordia (Dewitte and Murray, 2003; Shani et al., 2006). The observed phenotypes in IMA overexpressing tomato lines and Arabidopsis MIF1 overexpressor (Hu and Ma, 2006) are quite similar and correspond to phenotypes of known hormone-deficient or hormone-insensitive mutants. As a conserved feature for plant MIF proteins, IMA is also involved in a multiple hormonal signalling pathway, since IMA overexpressors are resistant to the effects of both auxin and cytokinin (Figure 2d). Interestingly, impairing the expression of IMA leads to increased cell divisions in the centre of the FM and the concomitant upregulation of ANT, known to stimulate cell proliferation through hormonal pathways (Mizukami and Fischer, 2000). As observed in Figure 2(d), we hypothesize that IMA may prevent the perception of hormonal signals, thus leading to an inhibition of cell divisions and hence meristem activity during FM termination. Therefore we cannot exclude the possibility that IMA may also regulate the D-function and SIWUS genes by modulating hormonal responses. Indeed the maintenance of meristem activity does require direct interaction between WUS and the cytokinin signalling machinery (Leibfried et al., 2005).

Dealing with the molecular mechanism by which IMA controls the meristem activity, it is likely that IMA per se is not able to bind to DNA to exert its inhibitory function. Indeed MIF proteins do not harbour the required homeodomain involved in DNA binding such as in Flaveria ZF-HD proteins (Windhövel et al., 2000). Through its ZF domain IMA may rather modify the activity of other regulatory factors by modulating the specificity or affinity of the target site, or by affecting potential interactions with other cofactors (Tan and Irish, 2006; Windhövel et al., 2000). Our current efforts to identify putative targets should provide a critical step to decipher the role of IMA in the regulatory pathway controlling meristem activity.

The nucleotide sequence of IMA reported in this paper has been submitted to the DDBJ/GenBank®/EBI Data Bank, with accession number AM261628.
Experimental procedures

Plant and growth conditions

Cherry tomato (Solanum lycopersicum Miller cv. WVA106) plants were grown either on soil in a greenhouse or on Murashige and Skoog medium (Murashige and Skoog, 1962), under a thermosto- od of 25°C/20°C and a photoperiod of 16/8 h (day/night).

Plasmid construction

The Pro35S:IMA and Pro35S:ima" constructs were respectively obtained by subcloning the complete IMA cDNA (accession no. AM261628) as a BamHI-XhoI fragment and a 400-bp SalI-BamHI fragment corresponding to the complete open reading frame (ORF) followed by 102 nucleotides (nt) of the 3' untranslated region (UTR) amplified with the following oligonucleotides: CTCCCTCGTGCCAGCACAAGCTTACC and CTCCTCCGATCCCAAGCTTCC- AAATAGG into the modified pPZP212 binary vector (Hajdukiewicz et al., 1994).

The IMA RNAi construct was obtained from the IMA cDNA by amplifying a 321-bp fragment which corresponds to 103 nt from the 3' and ORF followed by 118 nt of the 3' UTR, using the following oligonucleotides: AAAAAGCAGGCTTGAGATATGTTGAGTGCGAG and AGAAAGCTGGTGTCACACCTTATTCACACACAG. The amplified DNA fragment was cloned using the cloning GATEWAY system (Clontech, http://www.clontech.com/) as described by Karimi et al. (2002), with pDONR™210 as the entrance vector and pK7GWIWG2(1) as the destination vector.

Plant transformation

The different constructs were introduced into Agrobacterium tumefaciens strain GV3101 pMP90 (Koncz and Schell, 1986) using electroporation (2.5 kV, 400 μF). The transformation of tomato cotyleods by agro-infection was performed as described (Cortina and Cullaıñez-Macia, 2004) to generate Pro35S:IMA, Pro35S:ima" and Pro35S:ima" transgenic plants.

In situ hybridization analysis

In situ hybridization of digoxigenin-UTP labelled RNA probe to mRNA was performed as described (Bisbis et al., 2006). To synthesize the riboprobes, specific cDNA fragments were amplified using the following combinations of forward and reverse oligonucleotides: GTGCAAGCTTGCTGAC and CACACCTTATTCCACACAC for IMA; CTGCCCAGCTTCGGAAGGACTGTTCTGGA for SIWUS (accession no. AJ538329); AAT/GAGGAGGAGGAGGATCCTGC and GTGCTAAAAATATATAGAAG for TAG1 (accession no. AY098735); GACTTGAAGGAGGTAC and CAGCTGTAACACTATAGAAG for S1A7T (accession no. B9127245). The cDNA fragments were then cloned into the pGEM-T easy vector (Promega, http://www.promega.com/). Antisense and sense control RNA probes were transcribed using SP6 and T7 RNA polymerase, respectively. Hybridization and signal detection were done according to Bisbis et al. (2006). To be as quantitative as possible, all samples originating from the different tomato lines were processed in the same way, at the same time and prepared on the same slide for in situ hybridizations.

Semi-quantitative RT-PCR and northern blot expression analyses

Total RNA was purified from tomato organs and dissected tissues using a plant RNeasy purification kit (Promega). For northern blot analyses, total RNA was size-fractionated by 6.6% (v/v) formaldehyde-1.2% (v/v) agarose gel electrophoresis, transferred to Hybond-N (GE Healthcare, http://www.gehealthcare.com/) membranes by capillarity and hybridized to random-primed labelled IMA cDNA probe. Hybridizations were performed at 65°C according to standard procedures.

The RT-PCR experiments and analyses were performed as described in Joubès et al. (2001), using the following IMA gene-specific primer combination: GAGTGAGATATGTTGAGTGAC and CACACCTTATTACACAC, and the described set of primers used to amplify specifically the cell cycle genes Solly; CycD31, Solly; CycB21, Solly;CDK1A1, Solly;CDK821 and Solly;KRP1 and the control gene Solly;Actin1.

Sample preparation, histological and cytological methods, and microscopy

For analysis of floral organs, floral buds were photographed under a Canon 300D digital camera (Canon, http://www.canon.com/). Digital images were processed using Adobe Photoshop software (http://www.adobe.com/).

For histological examination, tissues were fixed, embedded in paraffin wax, sectioned and stained as described (Bereteerbide et al., 2002). For the determination of carpel wall cell layer number, carpels of opening flowers were fixed in 4% (w/v) formaldehyde, 50% (v/v) ethanol, 5% (v/v) acetic acid for 4 h at room temperature (22°C) as described by Chenici et al. (2005). Student’s t-test was used to evaluate the significance of the results.

For analysis of ovules and derived structures, samples were dissected from tomato berries, fixed for 30 min in 50% (v/v) ethanol/ 50% (v/v) acetic acid, rehydrated and then incubated overnight at 4°C in a clearing solution (8 g chloral hydrate, 11 ml water, 1 ml glycerol). For observation, samples were mounted in a drop of a clearing solution and photographed under a Zeiss Axiosplan microscope (http://www.zeiss.com/) with differential interference contrast optics using a SPOT-RT Color camera (Diagnostics Instruments Inc., http://www.diaginc.com/).

Scanning electron microscopy was performed on flowers that were fixed overnight in 3% glutaraldehyde in 0.025 M sodium phosphate (pH 7.0) at 4°C. After rinsing and dehydration in a graded ethanol series, they were critical point dried in liquid carbon dioxide. Individual buds were coated with gold, and viewed with a scanning electron microscope (Philips, http://www.philips.com/) at an accelerating voltage of 3–10 kV.

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Supplementary Material

The following supplementary material is available for this article online:

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Figures S1. Comparative analysis of the expression of IMA and SIMIF1-like genes in tomato vegetative and mature floral organs by RT-PCR.

Figures S2. Molecular analysis of IMA overexpressor, antisense and RNAI transgenic lines.

Figures S3. Phenotypic analysis of IMA overexpressor transgenic lines.

Figure S4. Relative mRNA abundance of cell cycle genes in isolated carpels of wild-type (WT), Pro35S:IMA4 and Pro35S:ima1 lines, determined by semi-quantitative RT-PCR.

Figure S5. TM29 C-type gene expression in Pro35S:ima1 and Pro35S:IMA4 plants.

Figure S6. Tentative model for the effects of IMA on meristem activity and ovule development.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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