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Hormonal control of the inflated calyx syndrome, a morphological novelty, in *Physalis*

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Summary

The 'Chinese lantern' phenotype or inflated calyx syndrome (ICS) – inflated sepals encapsulating the mature berry of *Physalis floridana* – is a morphological novelty within the Solanaceae. ICS is associated with heterotopic expression of *MPF2*, which codes for a MADS-box transcription factor otherwise involved in leaf formation and male fertility. In accordance with this finding, the *MPF2* promoter sequence differs significantly from that of its orthologue *STMADS16* in the related *Solanum tuberosum*, which does not exhibit ICS. However, heterotopic expression of *MPF2* is not sufficient for ICS formation in *P. floridana* – fertilization is also important. Here we report that the hormones cytokinin and gibberellin are essential for ICS formation. MPF2 controls sepal cell division, but the resulting cells are small. Calyx size increases substantially only if gibberellin and cytokinin are available to promote cell elongation and further cell division. Transient expression of appropriate MPF2-/STMADS16-GFP fusions in leaf tissues in the presence of hormones revealed that cytokinin, but not gibberellin, facilitated transport of the transcription factor into the nucleus. Furthermore, an ICS-like structure can be induced in transgenic *S. tuberosum* by ectopic expression of *STMADS16* and simultaneous treatment with cytokinin and gibberellin. Strikingly, transgenic Arabidopsis ectopically expressing solanaceous MPF2-like proteins display enhanced sepal growth when exposed to cytokinin only, while orthologous proteins from non-solanaceous plants did not require cytokinin for this function. These data are incorporated into a detailed model for ICS formation in *P. floridana*.

Keywords: inflated calyx syndrome (ICS), morphological novelty, MADS-box gene *MPF2*, plant hormone, cytokinin, gibberellin, *Physalis*, Solanaceae.

Introduction

Deciphering the molecular mechanisms underlying the origins of morphological novelties is one of the primary challenges in evolutionary biology. The few cases studied thus far in animals (Keys *et al.*, 1999; Lee *et al.*, 2003), as well as in plants (Frary *et al.*, 2000; Wang *et al.*, 1999), suggest that heterochronic or heterotopic expression of pre-existing functions, rather than the 'invention' of new functions, is the key event (He and Saedler, 2005; Hileman *et al.*, 2003; Kanno *et al.*, 2003; Wang *et al.*, 1999).

In angiosperms, flower structure is an attractive target for evolutionary studies. Flowers consist predominantly of four concentric whorls of organs. In the innermost circle are the female sexual organs, the carpels containing the ovules. The male organs, the stamens in which pollen is produced, form the next whorl. Petals, often colourful, surround the sexual organs, and the mostly green sepals follow in the outermost whorl. The last two whorls comprise the perianth, which assumes different functions during flower development. The sepals form the calyx, which is recruited for photosynthesis (Herrera, 2005) or protects the developing flower buds against adverse biotic and abiotic effects, such as insect damage and temperature changes. Transcription factors belonging to the MADS-box class play a prominent part in the determination of organ identity in flowers.

Species richness in the angiosperms is often associated with diversification of perianth organs, which may involve changes in organ identity, number of whorls, coloration, number, size and form of organs within a whorl, and variation in form and symmetry of the whorls. Morphologi-

cal novelties can affect any of these traits and may change the floral structure considerably (He *et al.*, 2004).

For example, in the Plantaginaceae, most species possess zygomorphic flowers with bilateral symmetry, and the evolution of a species with radially symmetrical (actinomorphic) flowers, as in *Mohavea*, has been shown to be due to heterotopic expression of a TCP transcription factor (Hileman *et al.*, 2003). Another example concerns a characteristic trait of the Liliaceae, which feature an entirely petaloid perianth: both whorls form petals, the so-called tepals. Heterotopic expression of class B MADS-box genes could have triggered this homeotic change of the first-whorl organs, as has been described for tulips (Kanno *et al.*, 2003). Indeed, Davies *et al.* (1996) were able to generate double flowers in transgenic *Nicotiana tabacum* by ectopically expressing class B MADS-box genes from *Antirrhinum majus*. Therefore, in the evolution of Liliaceae, heterotopic expression of a class B MADS-box gene could have played an important role.

The Solanaceae is a family rich in flower and fruit diversity (Knapp, 2002; Knapp *et al.*, 2004). Some genera, including *Physalis*, feature a morphological novelty known as the 'Chinese lantern' or inflated calyx syndrome (ICS; He *et al.*, 2004). While *Solanum tuberosum* has small sepals throughout floral and fruit development, *Physalis floridana* forms small sepals during flower development, but these increase in size and modify their architecture upon pollination/fertilization, ultimately forming the characteristic Chinese lantern surrounding the mature berry (He and Saedler, 2005; He *et al.*, 2004). Heterotopic expression of the MADS-box transcription factor MPF2, together with a pollination-dependent signal, is essential for ICS formation in this species (He and Saedler, 2005).

In the work reported here, *S. tuberosum*, *P. floridana* and *Arabidopsis thaliana* were used to study the nature of this signal. We show that the plant hormones cytokinin and gibberellin both play a role in ICS formation. While the effect of gibberellin on sepal cell elongation might be indirect, cytokinin controls sepal cell division together with MPF2 by enhancing the uptake of MPF2 into the nucleus. These findings are supported by our demonstration that an ICS-like structure can be induced in *S. tuberosum* through ectopic expression of *STMADS16* (the *S. tuberosum* orthologue of *MPF2*) in the presence of cytokinin and gibberellin. These data are incorporated into a model for ICS formation.

Results

Formation of the 'Chinese lantern' or ICS requires heterotopic expression of the MADS-box transcription factor MPF2, as well as signals that are dependent on pollination. MPF2 itself has been shown to promote cell division and to inhibit cell elongation in the calyx of the self-fertilizing *P. floridana*, as well as in transgenic *S. tuberosum* (He and Saedler, 2005). As developing seeds are known to produce hormones such as cytokinins and gibberellins, which influence cell division and/or cell size (Kende and Zeevaart, 1997; Ozga and Reinecke, 2003; Weyers and Paterson, 2001), we suspected that one or both of these hormone types might be involved in ICS formation.

Crosstalk between calyx and developing fruit (berry) during ICS formation

Ablation experiments involving the removal of sepals, pistils, stamens or developing fruits suggested that crosstalk occurs between the sepals and the developing fruit during ICS formation. Preventing fertilization by emasculation (data not shown) or by removal of the stigma/style at the flower-bud stage inhibited further calyx formation, while ablation of the fertilized carpel soon after pollination abrogated sepal growth and therefore prevented ICS formation (Figure 1a(i), column 2). However, if the developing berry was removed when the sepal was 1 cm long, ICS formation still occurred, although the resulting structure was smaller than the wild type [Figure 1a(i), column 3; cf. sepal length in untreated wild type in column 1]. This suggests that pollination or early steps in fertilization provide substances – signals or nutrients – for sepal growth. On the other hand, ablation of sepals <1 cm long prevented fruit development [Figure 1a(ii), columns 1–3], while ablations done at later stages allowed the development of smaller berries (Figure 1a(ii), columns 4–6), suggesting that sepals also contribute to fruit development, as in the case of *Helleborus foetidus* (Herrera, 2005).

Thus in *P. floridana* signals (and/or nutrients) appear to move in both directions – from berry to sepals and *vice versa* – and we set out to determine the nature of these signals. Developing fruits, for example, are known to produce auxins, cytokinins and gibberellins (Ozga and Reinecke, 2003; Weyers and Paterson, 2001). We chose to focus on these, and their effects on ICS development were analysed further.

Cytokinin- and gibberellin-induced signal(s) are essential for ICS formation

While wild-type *P. floridana* displays the fully developed ICS (Figure 1b, first picture; for quantification see first column from the left), ablation of stigma/style of flower buds completely prevents ICS formation. Furthermore, neither mock treatment with water nor the addition of auxin restored the syndrome (Figure 1b, second picture and blue columns), and such flowers ultimately died. In contrast, treatment of stigma/style ablated flower buds with a cytokinin solution (6-benzylaminopurine, 6-BAP, grey column) or a gibberellin solution (gibberellic acid 3, GA3, red column) restored ICS formation (Figure 1b, third and fourth pictures). The tiny berries that developed in these 'lanterns', however, did not contain any seeds.

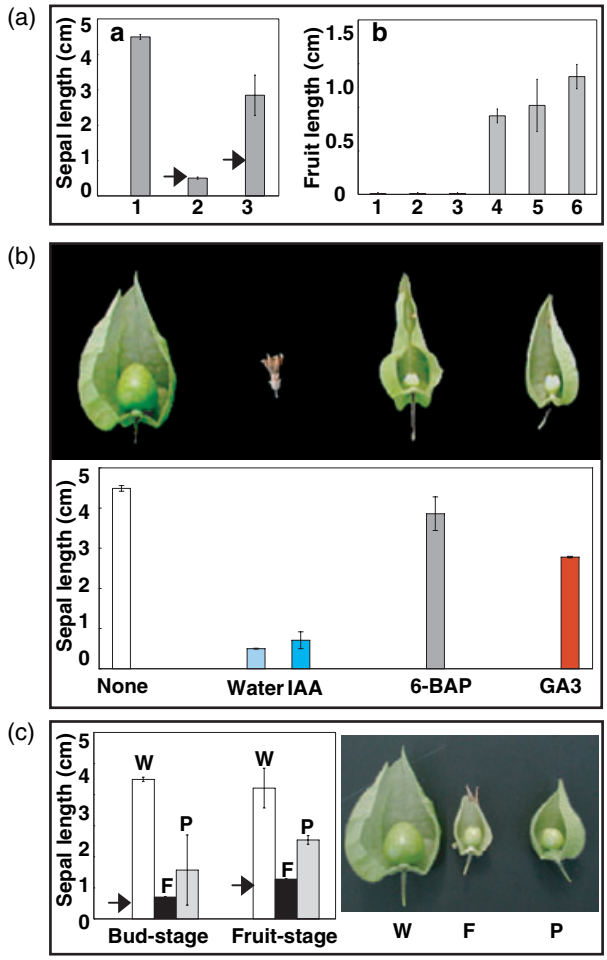

Figure 1. Dissection of signals directing post-floral calyx development in *Physalis*.

(a) Crosstalk between calyx and developing fruit as revealed by ablation experiments.

(i) In flower buds with sepals approximately 0.5 cm long, stigma/style was ablated or developing fruits were removed when sepal length was approximately 1 cm (black arrows); sepal lengths were measured at maturity. 1, unperturbed calyx development; 2, stigma/style-ablated flower buds; 3, developing fruit removed. Error bars, SD.

(ii) Fruit development after removal of calyx. (1–3) Ablation of sepals from flower buds (1), mature flowers (2) and developing fruits with sepals <1 cm (3). (4–6) Ablation of sepals >1 cm. Error bars, SD.

(b) Hormone treatment of stigma/style-ablated flower buds.

Upper panel, inflated calyx syndrome (ICS) phenotypes obtained following treatments indicated. Part of the calyx was removed to expose the developing berry. Note that use of indole-3-acetic acid (IAA) or water does not restore calyx growth. Lower panel, average sepal lengths obtained under different treatments. Error bars, SD.

(c) Inhibitors of gibberellic acid (GA3) biosynthesis also affect calyx development.

Left panel: quantification of sepal length. Arrows indicate times when treatments were initiated: bud stage (sepal length approximately 0.5 cm) and fruit stage (sepal length approximately 1 cm). Open columns, lengths of mature sepals treated with water only. Filled and shaded columns give sepal lengths obtained following treatment with flurprimidol (F) and paclobutrazol (P), respectively. Right panel: phenotypes observed after these treatments at the fruit stage. Part of the calyx was removed to expose the developing berry. Further details in Experimental procedures. Error bars, SD.

The involvement of gibberellins in ICS formation was corroborated by inhibiting gibberellin biosynthesis in floral buds of wild-type *P. floridana* with paclobutrazol and flurprimidol (Figure 1c). When flowers were treated at the bud stage with flurprimidol, no ICS developed (Figure 1c, filled column). Treatment with paclobutrazol at bud stages resulted in a wide range of calyx phenotypes, from no ICS to small 'lanterns' (see SD in Figure 1c, shaded column). This variability was eliminated if treatment was delayed until the sepals were 1 cm long (Figure 1c, shaded column). Given that cytokinin and gibberellins are involved in ICS formation, the question arose whether these hormones trigger and/or sustain sepal growth in the presence of MPF2 during ICS formation.

A single exposure to hormones is sufficient to cause calyx and sepal cell expansion

The sepals of flower buds and flowers of *P. floridana* remain small until fertilization has occurred and the berry starts to develop, at around 6–7 days post-anthesis under glasshouse conditions. At this time, growth of the calyx resumes (Figure 2; He and Saedler, 2005). Interestingly, sepal cells of *Physalis* flower buds are very small, and they elongate only slightly during flower development (Figure 2a). However, post-pollination – during fruit development – organ and cell length increase by three- to fourfold (Figure 2a, 14-day column).

As treatment of the calyx with hormones can mimic the effect of fertilization on calyx growth (Figure 1), we suspected that cytokinin and gibberellin might cause elongation of the small cells observed in immature flowers (Figure 2a). This is indeed the case. The response to hormone treatment appeared to be twofold and to change during development. After 3 days' treatment there was no significant increase in cell size compared with the untreated control, but the sepals had doubled in length – compare the black columns at day 3 for treated and hormone-treated panels (cell size), and white/green columns (organ size) in Figure 2(a). This finding suggested that hormone treatment promotes cell division. By day 7, not only sepal length but also sepal cell length had tripled, indicating that hormone treatment also affects cell elongation (Figure 2a, epidermal cell sizes). This clearly shows that the calyx of a *P. floridana* flower has the potential to increase in size even before pollination, provided the appropriate hormones are available.

Figure 2(b) shows the response of sepal length to diverse regimes of hormone treatment. A single treatment (dotted green line) with the hormone mixture (GA3 and 6-BAP) is sufficient to induce the formation of a lantern comparable in size with those seen under the natural conditions of self-fertilization (black line). However, unlike the latter, which persist for several weeks (Figure 2b), the induced structures dropped off after day 18. Repeated hormone treatments

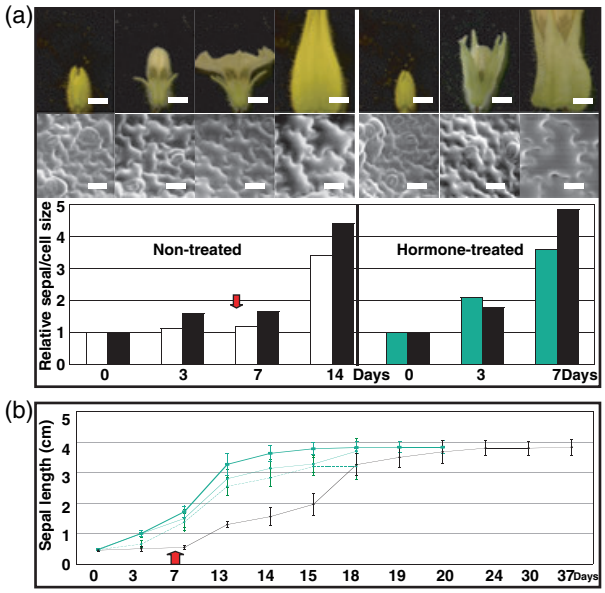

Figure 2. Hormones trigger inflated calyx syndrome (ICS) formation before pollination and sustain ICS maturation in *Physalis*.
(a) ICS results from cell division and cell expansion; both processes are controlled by plant hormones.
Upper section, sepal phenotypes observed in control (non-treated) plants and plants treated with hormones following stigma/style ablation of flower buds (scale bars, 0.25 cm), with SEM pictures of calyx epidermis at the transition zone from the lobe to the gamophylletic part of the sepals (scale bars, 25 μm). Lower section, relative sepal length and cell size in untreated and hormone-treated plants at times indicated. Stages of development are given in days (d) after sepals reached a length of 0.5 cm. Open columns, relative sepal length in non-treated conditions; green columns, sepal length after treatments with a mixture of GA3 and 6-BAP; black columns, relative cell sizes. For relative quantifications, sepal length and cell size in untreated plants at day 0 were arbitrarily set to 1. In all, 561 cells of non-treated calyces were measured at day 0, 337 at day 3, 237 at day 7, 289 at day 14. For hormone-treated calyces, 374 cells were measured at day 3 and 195 at day 7. Red arrow, approximate time of pollination.
(b) A single treatment with hormones is sufficient to trigger ICS formation. Black line, sepal growth under non-treated conditions; green lines, growth response of sepals that develop from depistillated buds exposed to various regimes of treatment with a mixture of GA3 and 6-BAP. Thick green line, results of daily treatment for 7 days; fine green line, daily treatment for 3 days; dotted line, a single treatment on day 0. Red arrow, approximate time of pollination. Error bars, SD.

(three daily doses, thin green line; seven daily doses, bold green line) extended the time to lantern loss without causing further organ growth. A single hormonal treatment is therefore sufficient to trigger calyx growth, but in order to maintain the developing lantern on the plant, hormone levels must be sustained.

Plant hormones can control gene expression (Bonhomme *et al.*, 2000; Estruch *et al.*, 1993; Schmülling *et al.*, 1997; Shakirova *et al.*, 2002), but they can also exert their regulatory effects at post-transcriptional levels (Downes and Crowell, 1998; Hare and van Staden, 1997; Nanjo *et al.*, 2004; Schmülling *et al.*, 1997). MPF2 is a key factor in ICS formation in *P. floridana* (He and Saedler, 2005), so we first looked for changes in the expression of *MPF2* in response to cytokinin and/or gibberellin using Northern analysis, but could detect no effect on the levels of *MPF2* transcripts (data not shown). Therefore neither pollination (He and Saedler, 2005) nor these plant hormones control *MPF2* transcription.

Based on previous work, MPF2 and its orthologue STMADS16 from *S. tuberosum* differ significantly in amino acid sequence at their C-terminus (He and Saedler, 2005). Therefore, these two proteins might respond differently to hormone treatment.

Cytokinin controls the intracellular localization of MPF2 and STMADS16

Gibberellin and/or cytokinin could exert their effects at the translational and/or post-translational level. Hormone-induced signal cascades might possibly alter MPF2 or its Solanum orthologue STMADS16, and thus affect their subcellular localization. We therefore tested the effect of the hormones on the intracellular localization of MPF2 and STMADS16. Physalis leaf or sepal epidermal cells were treated, or not, with the hormones 6-BAP and/or GA3, and were co-transformed by particle bombardment, with a mixture of plasmids carrying constructs encoding the translational fusion 35S::SPL8-RFP and either 35S::MPF2- or STMADS16-GFP. SPL8 codes for a transcription factor (Unte *et al.*, 2003) previously shown to be present in the nuclei (Birkenbihl *et al.*, 2005). Thus the red fluorescence due to SPL8-RFP was used as an internal control to indicate the position of the nucleus. The cellular localization of MPF2 or STMADS16 could be determined from its green fluorescence due to the translational fusion to GFP.

Figure 3 shows superimposed pictures of red and green fluorescence in leaf cells transformed with the various plasmids. Co-localization of SPL8 and MPF2 or STMADS16 can be seen (yellow signal) when red and green signals are superimposed (Figure 3a–f). Note that significant amounts of MPF2 or STMADS16 were found in the nucleus only when the cells were treated with cytokinin (Figure 3b,e). Cells exposed to gibberellin (Figure 3c,f) or not treated with hormones (Figure 3a,d) showed mostly red nuclei characteristic for SPL8 localization, while most of the MPF2 or STMADS16 was found in the cytoplasm as revealed by the green fluorescence, although some MPF2 and STMADS16 is detectable in the nucleus as well. The results of these experiments are summarized in Figure 3(g), in which the percentage of cells showing nuclear localization of MPF2 and STMADS16 is given. Figure 3(h) shows the ratio of the amounts of the two transcription factors in nuclear versus cytoplasmic compartments. Both measurements show clearly that cytokinin facilitates the transport of MPF2 and STMADS16 proteins into the nuclei of the cells (Figure 3g,h, columns 2), indicating that the divergence of the C-termini of these proteins does not affect transport to the nucleus.

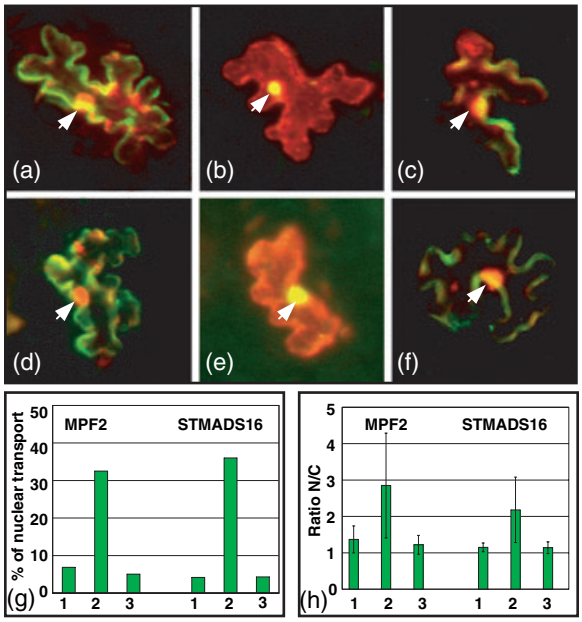

Figure 3. Cytokinin affects the subcellular localization of MPF2 and STMADS16.
Physalis leaves were bombarded with mixtures of plasmids encoding either SPL8-RFP and MPF2-GFP (a–c) or SPL8-RFP and STMADS16-GFP (d–f). Epidermal cells were either left untreated (a, d) or treated with 6-BAP (b, e) or GA3 (c, f), respectively. White arrows, nuclei.
(g) Percentage of cells showing a nuclear GFP signal: 1, non-treated; 2, BAP; 3, GA3. Numbers of cells examined were 73 (1), 83 (2), 79 (3) for MPF2-GFP; and 72 (1), 75 (2), 70 (3) for STMADS16-GFP.
(h) Relative nuclear transport is expressed in terms of the ratio of GFP signals in the nucleus versus the cytoplasm (ratio N/C): 1, non-treated; 2, BAP; 3, GA3. Numbers of cells analysed were 27 (1), 37 (2), 26 (3) for MPF2-GFP; and 38 (1), 46 (2), 15 (3) for STMADS16-GFP. Error bars: SD.

Similar results were obtained (data not shown) using the above constructs in bombardment experiments with leaf and sepal tissues of *S. tuberosum* and leaf tissues of *A. thaliana*.

Given that heterotopic expression of *MPF2* in the calyx, in the presence of a simple hormone mixture, is sufficient to produce ICS in *P. floridana*, it should be possible to induce the syndrome in a species that does not normally display it, such as *S. tuberosum*.

ICS formation in transgenic S. tuberosum

Solanum tuberosum has small sepals throughout flower and berry development. Unlike *MPF2* in *Physalis*, which is expressed throughout vegetative and floral stages of development, expression of its orthologue *STMADS16* in potato is restricted to the vegetative phase of plant development (Garcia-Maroto *et al.*, 2000; He and Saedler, 2005). We generated transgenic *S. tuberosum* plants ectopically expressing either *MPF2* or *STMADS16*, and these were found to show both vegetative (Table 1) and floral phenotypes (Figure 4).

Four clonal plants each of wild-type *S. tuberosum* and transgenic *S. tuberosum* plants expressing *35S::MPF2* or *35S::STMADS16* were grown, and the vegetative parameters listed in Table 1 were measured. Leaf size was not affected, but the overall height of the transgenic plants was reduced, as was the average internode length and epidermal cell size in the stem. The number of cells per internode was increased, however, so that transgenic plants overexpressing *MPF2* or *STMADS16* had more but smaller internode cells than the wild type. Therefore, when overexpressed, both MPF2 and STMADS16 also promote cell division and repress cell elongation in vegetative parts of transgenic plants.

We previously showed that transgenic *S. tuberosum* plants ectopically expressing either *MPF2* or *STMADS16* develop slightly larger calyces than the wild type, and on fertilization these increased further, although curling outwards (He and Saedler, 2005). However, sepal growth was limited and certainly did not attain the size or the structure of the ICS in *P. floridana* (Figure 1), suggesting that creation of an ICS-like structure in *S. tuberosum* might be feasible if sufficient amounts of hormones were available. Treatment of transgenic *S. tuberosum* plants expressing *STMADS16* from the 35S promoter with cytokinin resulted in larger sepals that curled outward [Figure 4a(iv)]. However, administration of the gibberellin GA3 resulted in a larger calyx with a lantern-like structure reminiscent of ICS [Figure 4a(v)]. This trait was enhanced if the sepals were treated with a mixture of cytokinin and gibberellin. Unlike the ICS of *Physalis*, the 'lantern-like' structure in the transgenic potato had elongated sepal teeth [Figure 4a(vi)].

Table 1. Vegetative phenotypes of transgenic *Solanum tuberosum* plants

	Phenotypes						
Genotypes	Flowering time (days)	Height (cm)	Internode number	Average internode length (cm)	Cell number	Cell length (μm)	Cell number per internode
Wild type	73.0 ± 3.6	154.5 ± 8.0	28.75 ± 1.3	5.4 ± 0.3	400	145.0 ± 41.6	370.1 ± 19.7
35S::MPF2	70.5 ± 5.8	137.8 ± 4.0	29.00 ± 0.8	4.8 ± 0.1	683	98. 9 ± 22.1	480.3 ± 9.7
35S::STMADS16	69.0 ± 6.1	115.3 ± 4.3	30.75 ± 1.0	3.8 ± 0.2	887	67. 1 ± 21.5	558.6 ± 26.5

Flowering time (days), height (cm), internode number, cell number and cell length (μm) were measured directly; average internode length and cell number per internode were calculated.

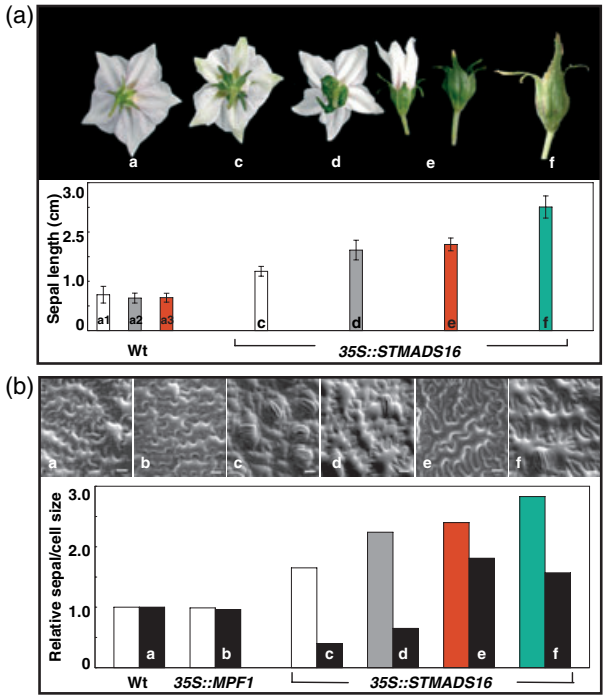

Figure 4. Expression of inflated calyx syndrome (ICS) in transgenic *Solanum tuberosum* lines.
(a) Analysis of transgenic *Solanum tuberosum* lines: *S. tuberosum* was transformed with various constructs that drive ectopic expression of *MPF1*, *MPF2* and *STMADS16*, respectively.
Upper panel: phenotypes of mature flowers and ICS-like structures. Wild-type (Wt) flower (i); *35S::STMADS16* transgenic flowers (iii); treated with 6-BAP (iv); GA3 (v); and a mixture of both hormones (vi). *35S::MPF1* plants treated with hormones did not show any phenotypic alteration with respect to Wt (not shown).
Lower panel: quantification of sepal lengths observed under the different hormone treatments. White columns, non-treated; grey, 6-BAP; red, GA3; green, 6-BAP and GA3. Numbers correspond to phenotypes shown in upper panel. Error bars, SD.
(b) SEM pictures of sepal surfaces and quantification of relative sepal lengths and cell sizes.
Upper panel: (i, iii, vi) as in (a); (ii) *35S::MPF1* transgenic line. Scale bar, 20 μm.
Lower panel: black columns, relative cell sizes observed under the various conditions tested; columns colour-coded as in (a). Quantification as described for Figure 2(a). Numbers of cells measured: 38 (i), 54 (ii), 89 (iii), 82 (iv), 21 (v), 31 (vi).

Transgenic *35S::MPF2 S. tuberosum* flowers treated with cytokinin or gibberellin also developed larger calyces but, irrespective of the hormone used, all sepals curled outward. Therefore only *35S::STMADS16* transgenic *S. tuberosum* lines were studied further.

Wild-type flowers [Figure 4a(i)], or flowers of transgenic plants overexpressing *MPF1* (data not shown), the closest paralogue of *MPF2*, and an orthologue of *STMADS11* of *S. tuberosum* previously used as a control to assess the specificity of MPF2 function (He and Saedler, 2005), did not respond to hormone treatment, thus confirming the specificity of STMADS16/MPF2 action. In summary, ectopic expression of *STMADS16*, the orthologue of *Physalis MPF2* in potato, and the availability of cytokinin and gibberellin are sufficient to allow an ICS-like structure to form in *S. tuberosum*.

Gibberellins control cell size in sepals of transgenic S. tuberosum *plants*

Gibberellins have been suggested to control cell size, while cytokinins are thought to be involved in cell division. Scanning electron micrographs of epidermal calyx tissues revealed a reduction in sepal cell size in *35S::STMADS16* transgenic plants (Figure 4b(iii), black column). As organ size had increased in these transgenics (Figure 4a(iii),b(iii), white column), STMADS16 appears to promote sepal cell division, resulting in larger numbers of small cells. Treatment with cytokinin resulted in a moderate increase in cell size and a further increase in calyx size, although the sepals now curled outward [Figure 4a(iv),b(iv)]. Administration of gibberellin, however, increased cell size substantially [Figure 4b(v)], while organ size increased only moderately [Figure 4a(v),b(v), red column]. The most striking effect was observed in experiments where plants were treated with a mixture of cytokinin and gibberellin. Cell size did not increase beyond the size observed on treatment with gibberellin alone [Figure 4b(vi)], but organ size was larger still [Figure 4a(vi),b(vi), green column].

Neither wild-type sepals [Figure 4a,b(i)] nor sepals of *35S::MPF1* transgenic flowers responded to hormone treatment [Figure 4b(ii)], supporting the specificity of the combination of MPF2/STMADS16 and hormone action on sepal cell division and sepal cell elongation, respectively, during formation of an ICS-like structure.

Effects of MPF2/STMADS16 in transgenic Arabidopsis thaliana

The phylogenetic relationship within the STMADS11 super-clade was established previously (He and Saedler, 2005). It was also shown that some MADS-box genes belonging to this super-clade, if expressed ectopically in transgenic Arabidopsis, induce a pleiotropic floral phenotype including secondary sepal growth (He *et al.*, 2004; Masiero *et al.*, 2004; Yu *et al.*, 2004). A typical example is the ectopic expression of the endogenous *AGL24* gene in *A. thaliana* [Figure 5, cf. (a) wild type and (e) transgenic line]. While several genes from non-solanaceous plants provoke such secondary sepal growth in Arabidopsis (He *et al.*, 2004; Masiero *et al.*, 2004), orthologues belonging to the *STMADS16* sub-clade from solanaceous species (Figure 5b), like solanaceous genes belonging to the *STMADS11* sub-clade, did not cause elongation of sepals in transgenic Arabidopsis plants (He and Saedler, 2005). However, in the present study we noted a striking difference between members of the two sub-clades in terms of their response to hormones, especially to cyto-

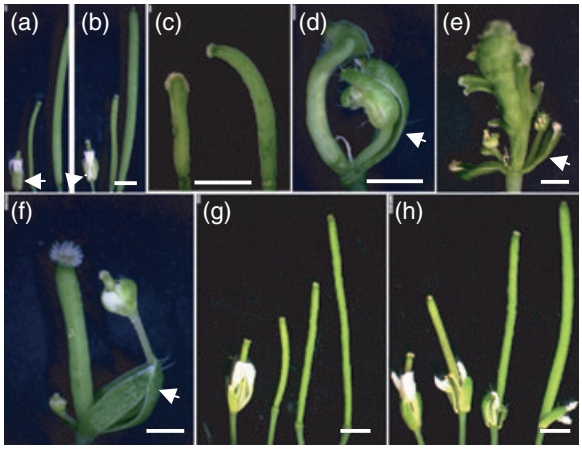

Figure 5. Cytokinin is required for solanaceous MADS function in transgenic Arabidopsis.
(a) Wild-type (wt) Arabidopsis thaliana.
(b) *35::MPF2* transgenic Arabidopsis line. The transgenic *35S::STMADS16* Arabidopsis line showed a similar phenotype (not shown).
(c) Wild-type Arabidopsis treated with the cytokinin 6-BAP.
(d, e) *35S::STMADS16* (d) and *35S::MPF2* (e) lines treated with 6-BAP.
(f) *35S::AGL24* transgenic line. Note that no change in sepal phenotype is observed when this line is treated with hormones (not shown).
(g, h) Wild-type (g) and *35S::MPF2* (h) line treated with GA3. *35S::STMADS16* line showed a similar phenotype to the *35::MPF2* line if treated with GA3 (not shown).
Scale bar, 0.25 cm; white arrows, sepals.

kinin. If *STMADS16* or *MPF2* was expressed ectopically in Arabidopsis, no elongated sepals were observed (Figure 5b) unless the transgenic plants were treated with cytokinin (Figure 5d,e). Aside from the many pleiotropic changes observed, sepal size (Figure 5, white arrows) increased substantially. No such effect was observed when wild-type plants were treated with cytokinin (Figure 5c). Note that the enhanced sepal growth seen in transgenic Arabidopsis expressing *AGL24* (Figure 5f) is not affected by hormone treatment. Treatment with gibberellin did not lead to such secondary sepal growth in wild-type (Figure 5g) nor in *MPF2* transgenic Arabidopsis plants (Figure 5h). In the latter case, an increase in longevity of the floral organs was observed such that they remained on the developing siliques (Figure 5h). Clearly, increased sepal size in the presence of MPF2 and 6-BAP is in line with the finding that this hormone facilitates nuclear transport of MPF2.

Discussion

Plant hormones play an important role in general plant development, including the pathways leading to the production of mature fruits and seeds (Ashikari *et al.*, 2005; Singh *et al.*, 2002). For example, auxins, gibberellins and cytokinins (among others) have been implicated in various stages of fruit development (Nitsch, 1970). While most ovary and ovule growth prior to pollination/fertilization is due to cell division controlled by endogenous hormones, during maturation of the ovary/ovule the level of these hormones drops and cell division ceases (Ozga and Reinecke, 2003). However, pollination/fertilization generates signals that initiate fruit growth. These signals are derived from pollen and/or produced by the ovary in response to pollen tube growth or to fertilization itself. Such signals include hormones such as auxins, gibberellins, cytokinins, and ethylene (Nitsch, 1970; O'Neill, 1997). Hence the development of fruits and seeds involves a complex interplay of cell division and differentiation under hormonal control, and in certain species the calyx (Ganelevin and Zieslin, 2002) might contribute to this.

In *Physalis*, we have shown that crosstalk occurs between the developing berry and sepals. If sepals are removed at early stages in development, no fruit is initiated. On the other hand, if fertilization is prevented, no further growth of the calyx is observed. The growing pollen tube, the fertilization process itself, or the developing fruit may provide signals needed for ICS formation. We have shown here that the cytokinin 6-BAP and the gibberellin GA3 can serve as signals that promote ICS formation.

A similar observation was reported previously for the Christmas rose, *Helleborus niger* (Salopek-Sondi *et al.*, 2002). The sepals of fertilized *H. niger* flowers grow, spread and turn green, and the peduncles elongate. Cytokinins and gibberellins stimulate growth and greening of the sepals, while gibberellin and auxin prompt peduncle growth. In *Physalis* cytokinins and gibberellins promote cell division and cell expansion, respectively, as in *Helleborus* (Salopek-Sondi *et al.*, 2002) and other cases (Fernie and Willmitzer, 2001; Matveeva *et al.*, 2004; Nishii *et al.*, 2004). In the present work, the effects of these hormones on the development of the morphological novelty ICS in *Physalis* were investigated at both cellular and molecular levels, and the results are discussed in the following sections.

Effects of cytokinin

Cytokinins and gibberellins can activate the expression of MADS-box genes, as demonstrated in the regulation of the floral transition in *Sinapis alba* (Bonhomme *et al.*, 2000). In *Physalis*, however, the expression of the MADS-box gene *MPF2* is not under the control of these hormones. They must therefore exercise their effects on calyx growth in other ways. Riou-Khamlichi *et al.* (1999) have provided evidence that cytokinin activates cell division in Arabidopsis via the induction of a D-type cyclin. In promoting cell division, cytokinins often act synergistically with auxins (Jacobs, 1997; Sieberer *et al.*, 2003). However, in *Physalis*, ICS formation could be triggered in (stigma/style) ablated flowers by exogenous application of cytokinin alone; no auxin was needed. Cytokinin, in conjunction with MPF2, did activate cell division. This was corroborated by the finding

that when transgenic *Solanum* and transgenic Arabidopsis plants that express *MPF2* or *STMADS16* ectopically are treated with cytokinin, sepal cell division is induced, leading to the formation of an ICS-like structure in the former and an increase in sepal size in the latter species. No effect of cytokinin on sepal growth was observed in the respective wild types.

A cytokinin-mediated signal cascade could possibly lead to modification of MPF2 or its interacting partners. Indeed, we found that in cytokinin-treated (but not in GA3-treated) cells, MPF2 was predominantly present in the nucleus. In *A. thaliana*, modification of the MADS-box protein AP1 has been described previously (Yalovsky *et al.*, 2000). That study suggested that farnesylation alters the function and perhaps the specificity of the transcription factor. The C-terminal sequence CFAA, a typical CaaX box, is apparently the target for the FTase. As the solanaceous MPF2-like proteins do not contain CFAA termini, farnesylation in response to hormone treatment seems unlikely. However, given its central role in the regulation of protein transport to the nucleus (Jans and Hubner, 1996), phosphorylation seems a more likely modification (Trewavas, 1976). Indeed, Fujita *et al.* (2003) have suggested that a meristimatic receptor-like kinase (MRLK) is needed for translocation of AGL24, the orthologue of MPF2, to the nucleus in Arabidopsis. They proposed that the RLK signalling pathway mediates phosphorylation of this MADS-box protein. Interestingly, only the modified AGL24 appears to be transported to the nucleus. The fact that excessive sepal growth seen in transgenic Arabidopsis plants ectopically expressing *AGL24* is not dependent on externally supplied cytokinin (Figure 5e) could be explained neatly if the MRLK system already operative in the plant modifies this transcription factor.

By analogy, MPF2, the orthologue from *Physalis*, might behave similarly in that its transport to the nucleus also requires phosphorylation. MPF2 was observed in the nucleus only if the tissue was treated with cytokinin. This hormone was recently shown to trigger phosphorylation of ARR2 in Arabidopsis (Kim *et al.*, 2006). Therefore such a modification of MPF2 and/or of its interacting partners is conceivable, and might facilitate transport into the nucleus. Whether MPF2 is indeed phosphorylated in response to cytokinin during ICS formation in *Physalis* remains to be seen, and the mechanism of its nuclear transport awaits elucidation.

An alternative possibility for cytokinin action is transcriptional control (Bonhomme *et al.*, 2000) of other MADS-box proteins that interact with MPF2. Heterodimerization could enhance nuclear uptake, as in the case of AP3 and PI in Arabidopsis (McGonigle *et al.*, 1996). Modification of the MPF2 protein itself or its partners might also affect its potential for dimer or oligomer formation and, depending on the composition of these complexes, this could account for its control of calyx cell division in *Physalis*.

Although various plant MADS-box proteins are known to control organ size and cell division (Davies *et al.*, 1999; Gu *et al.*, 1998; Meyerowitz, 1997; Rosin *et al.*, 2003), no details have been reported thus far concerning the mechanism by which these MADS-box proteins produce their effects. On the other hand, one of the founding members of the MADS-box class, MCM1 from yeast, is known to form ternary complexes with Forkhead transcription factors, and thus interacts directly with cell-cycle control (Bähler, 2005).

A differential search for MPF2 interactors in the presence and absence of cytokinin, known to be involved in cell cycle control, and an analysis of MPF2 target genes, might provide further insight into how MPF2, in conjunction with cytokinin, controls cell division during ICS formation in *Physalis*.

Interestingly, while the MADS-box protein AGL8/FUL inhibits cell division and promotes cell expansion in the valve of Arabidopsis (Gu *et al.*, 1998), MPF2 does the opposite in the calyx of *P. floridana*, promoting cell division and inhibiting cell elongation (He and Saedler, 2005).

The effect of gibberellin

Gibberellins control many aspects of plant growth and development, including the transition from vegetative growth to flowering (Swain and Singh, 2005). In *Lemna minor*, for example, GA3 was shown to control root growth by regulating cell elongation (Inada and Shimmen, 2000). In Arabidopsis, gibberellins promote the destruction of DELLA, which acts as a brake on cell proliferation and enlargement (Achard *et al.*, 2006; Fu *et al.*, 2004). In stigma/style-ablated *Physalis* flowers, calyx growth resumes after exogenous application of GA3 to permit full development of ICS. Conversely, application of inhibitors of GA3 biosynthesis inhibits ICS formation. This demonstrates that GA3 is also involved in ICS development. Independent support for this idea comes from transgenic *Solanum* overexpressing *STMADS16.* When such flowers were treated with GA3, an ICS-like structure was formed, predominantly due to enlargement of calyx cells. Overexpression of *STMADS16* alone led to a slightly larger calyx consisting of only small cells.

How does gibberellin mediate its function in ICS formation? GA3 could increase cell size in the calyx either by degradation of DELLA or by modifying the MPF2 protein, or it could raise the amount of MPF2 protein. Translational control of α-amylase levels by gibberellins was observed previously in germinating rice seeds (Nanjo *et al.*, 2004). As no MPF2-specific antibodies are available, we currently cannot exclude post-transcriptional control. Gibberellin-mediated modification of MPF2 appears less likely, as gibberellin treatment had no effect on nuclear transport of MPF2. This leaves the first option: gibberellin-dependent

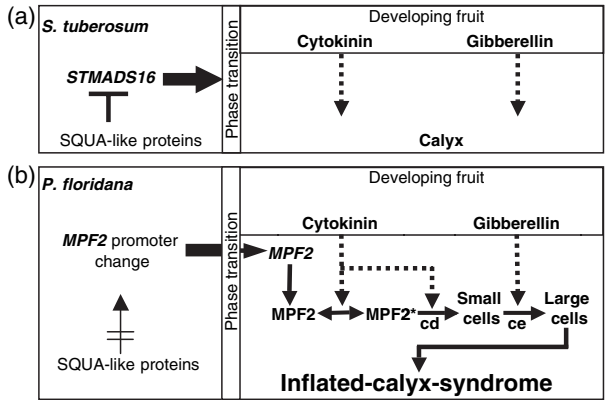

Figure 6. Model for formation of inflated calyx syndrome (ICS) in *Physalis floridana*.
A molecular scenario that explains the divergent course of calyx development in *Solanum tuberosum* (a) and *Physalis floridana* (b) is given. For details see text. cd, Cell division; ce, cell expansion. Star indicates a modified (e.g. phosphorylated) form of the MPF2 protein. Dashed lines highlight main findings of this study.

destruction of DELLA. In this case, the gibberellin effect on ICS formation of *Physalis* could be indirect.

A model for ICS formation

The data described here are summarized in Figure 6, which shows a comparison between formation of the normal calyx in *S. tuberosum* and the ICS in *P. floridana*.

In *S. tuberosum*, *STMADS16* is expressed only in vegetative tissues of the plant, possibly because a SQUA-like protein can bind to its promoter and thus arrest its expression in floral organs (Garcia-Maroto *et al.*, 2000; He and Saedler, 2005; Huijser *et al.*, 1992; Mandel *et al.*, 1992; Vrebalov *et al.*, 2002; Yu *et al.*, 2004). Mutation of the orthologue of *Antirrhinum majus SQUA* (Huijser *et al.*, 1992) in tomato, *LeMADS-MC*, results in large leaf-like sepals (Vrebalov *et al.*, 2002), indicating that SQUA-like proteins control calyx development in this solanaceous plant. An *STMADS16-like* gene might be its target. The promoters of *STMADS16* of *S. tuberosum* (Garcia-Maroto *et al.*, 2000) and *AGL24*, its orthologue in *A. thaliana* (Yu *et al.*, 2004) each contain binding sites for MADS-box transcription factors (He and Saedler, 2005), so-called CArG boxes (Shore and Sharrocks, 1995). Constitutive expression of *AGL24* in *ap1* Arabidopsis flowers generates large bract-like sepals (Figure 5f; Mandel *et al.*, 1992; Yu *et al.*, 2004). This suggests that AP1, the orthologue of SQUA, represses the expression of *AGL24* during the transition from vegetative to generative growth. In *S. tuberosum*, therefore, a SQUA-like protein could bind to the CArG-boxes (West *et al.*, 1998) of the *STMADS16* promoter and thus repress its expression in the floral program, as indicated (Figure 6a). On fertilization by compatible pollen, berries develop and gibberellins and cytokinins are probably produced, but as no STMADS16 protein is available in the calyx, no further sepal growth is observed. However, an ICS-like calyx is formed in potato (Figure 4f) if (i) STMADS16 protein is provided via ectopic expression in floral organs, and (ii) an external source of the plant hormones cytokinin and gibberellin is supplied.

The situation in *Physalis* is different (Figure 6b). Calyx growth resumes on pollination, thus generating the characteristic Chinese lantern. Heterotopic expression of *MPF2* in floral organs of *Physalis* is due to alterations in the *MPF2* promoter. This promoter does not contain binding sites for a SQUA-like protein (He and Saedler, 2005), which therefore cannot repress *MPF2* expression during the transition phase. Thus *MPF2* transcripts are available in floral organs and some protein may be formed, which enters the nucleus (Figure 3a) and thus stimulates cell division to generate small cells [Figure 4b(iii)]. Previously we showed that MPF2 promotes cell division but inhibits cell elongation (He and Saedler, 2005). Cytokinin-mediated modification of MPF2 facilitates its transport to the nucleus (Figure 3b) and thereby stimulates cell division [Figure 4b(iv)]. Gibberellin is primarily responsible for elongation of the resulting small cells [Figure 4b(v)] and thus contributes significantly, although indirectly, to ICS formation.

Both gibberellins and cytokinins are naturally provided on pollination/fertilization. Therefore the rate-limiting step in the evolution of ICS is the availability of an MPF2-like protein in calyx cells. This is achieved by changes in the promoter of the *MPF2* gene in the case of *P. floridana* (He and Saedler, 2005), and by changes in a SQUA-like transacting regulator, as in the tomato mutant *lemads-mc* (Vrebalov *et al.*, 2002) and in *ap1* mutants of Arabidopsis (Mandel *et al.*, 1992; Yu *et al.*, 2004).

Experimental procedures

Plant materials

The plant materials used in this study were: diploid *Physalis floridana*, and tetraploid *Solanum tuberosum* (Desirée) and *Arabidopsis thaliana* Col-1. Although many species of *Physalis* are self-incompatible (Igic *et al.*, 2006), *P. floridana* is self-compatible (He and Saedler, 2005). *Solanum tuberosum* (Desirée) can self provided proper pollinators are available. All lines were grown in the glasshouse of the MPIZ in Cologne.

Ablation experiments in Physalis

Emasculations and removal of stigma/style were done at bud stages (prior to pollination) when sepal length was approximately 0.5 cm. Developing fruits were removed carefully when the calyx was about 1 cm long. To allow access to developing fruits, the calyx was incised longitudinally and partially removed. Calyces were removed at different developmental stages (buds, flowers and developing fruits) as indicated. In each case, at least 10 samples were treated and analysed.

Hormone treatments of Arabidopsis, Physalis *and* Solanum

F_2 and F_3 transgenic Arabidopsis plants, as well as wild-type plants grown under long-day conditions on emergence of flower buds, were sprayed with 10 μM solutions of GA3 or 6-BAP daily for 7 days. Hormone solutions (10 μM GA3, 6-BAP or indole-3-acetic acid, IAA) were applied once a day to the sepals of five to 10 stigma/style-ablated flower buds of *P. floridana* using cotton buds. Treatments were continued for up to 1 week. Saturated aqueous solutions of paclobutrazol and flurprimidol, which inhibit gibberellin biosynthesis, were applied with cotton buds to 10 intact flower buds and 10 flowers bearing developing fruits at stages when calyces were approximately 0.5 or 1 cm long, respectively. Treatment was continued for 7 days. Tissues treated with water (or left untreated) served as controls. Flower buds of wild-type and transgenic *Solanum* plants were treated daily for 7 days with 10 μM GA3 or 10 μM 6-BAP, or a mixture of both hormones.

Northern blot analysis

Total RNA was isolated from treated and untreated *Physalis* floral organs at the times indicated in Figure 2(a) using the total RNA reagent kit (Biomol, http://www.biomol.com). Hybridization procedures and probe labelling were performed as described previously (He *et al.*, 2002).

Generation of transgenic plants

Construction of expression vectors, transformation and confirmation of transgenic *Solanum* lines were carried out as described previously (He and Saedler, 2005).

For Arabidopsis transformation, the full-length (sense) cDNAs of *AGL24*, *STMADS16* and *MPF2* were cloned into pRT100, then shuttled into the plant binary vector pBAR-A for expression from the CaMV 35S promoter. Transformation was mediated by GV3101 strains of *A. tumefaciens* using the floral-dipping protocol (Clough and Bent, 1998) with minor modifications. After seed germination, transgenic Arabidopsis containing the above constructs were screened by spraying with 0.15–0.2% Basta twice a week. Their genetic structure was confirmed by PCR and sequence analysis using gene-specific primers.

Phenotyping and quantification

Plant tissues were photographed using a digital camera (Nikon www.nikon.com) or the camera attached to a Leica microscope, and sepal and petal lengths of *Physalis* (five to 10 samples) and *Solanum* (25–30 samples) were measured. The following parameters of vegetatively propagated wild-type and transgenic *Solanum* plants (four of each per sample) were recorded: height below the first inflorescence, internode number and length, and time to flowering. The data were processed and graphs were generated using Microsoft EXCEL.

Scanning electron microscopy

Calyces from *Physalis*, wild-type *Solanum* and transgenic *Solanum* lines were collected, as well as stems from the same developmental stages, after flowering. For SEM observations, the harvested fresh materials were frozen in liquid nitrogen, sputter-coated with gold and examined using a DSM940 digital scanning microscope (Zeiss). Calyx cell sizes were quantified using the image-processing program IMAGEJ (http://rsb.info.nih.gov/nih-image; Abramoff *et al.*, 2004). Relative cell lengths in stems from wild-type and transgenic *Solanum* were measured at the same magnification. Graphs were plotted as described above.

Transient expression, fluorescence microscopy and quantification of nuclear transport

Full-length *MPF2* and *STMADS16* cDNAs expressed from the 35S promoter were cloned into the expression vector pGJ619 using the *Nco*I cloning site, to generate green fluorescent protein (GFP) fusion proteins. SPL8 from Arabidopsis was fused to red fluorescent protein (RFP) and used as an indicator for the nucleus (Birkenbihl *et al.*, 2005). Mixtures of SPL8-RFP with different GFP fusion constructs were delivered via particle bombardment (Bio-Rad, http://www.bio-rad.com) to leaves and sepals from *Physalis* and *Solanum* and to Arabidopsis leaves for transient expression studies. The plant materials were laid out on 0.8% agar plates supplemented with either 10 μM BAP or 10 μM GA3. Bombardment with DNA-coated gold particles was performed with a Biolistic PD-1000/He particle delivery system (Bio-Rad). Observations were made using a fluorescence microscope equipped with different filter sets, and digital pictures were taken using a camera operated by the DISKUS software package. Cells that showed both fluorescent signals were analysed further. The experimental procedures were as described previously (Jach *et al.*, 2001). Quantification of the ratio of nuclear to cytoplasmic fluorescence was carried out as described by Birkenbihl *et al.* (2005).

Primers and DNA sequencing

The primers used were commercially synthesized by Metabion International AG (http://www.mymetabion.com). Their sequences are available on request. All constructs were sequenced before transformation and bombardment experiments at the Automatic DNA Isolation and Sequencing Unit of the Max-Planck-Institute for Plant Breeding Research (Cologne, Germany).

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