The tomato NAC transcription factor \textit{SlNAM2} is involved in flower-boundary morphogenesis

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Abstract

Being composed of several whorls of distinct floral organs, the flower is one of the most complex organs in the plant. As such, the formation and maintenance of boundaries that separate the meristem from the floral organ primordium and adjacent organs are critical for its normal development. In \textit{Arabidopsis}, the miR164-regulated \textit{NAM} genes play key roles in floral-boundary specification. By contrast, much less is known about floral-boundary establishment in the model crop tomato. It was found that the miR164-regulated \textit{NAM} gene \textit{GOBLET} is expressed in the floral meristem–organ boundaries and its loss-of-function mutant produces flowers with fused organs, indicating its requirement for tomato floral-boundary formation. It was found here that sly-miR164 targets the transcripts of three additional uncharacterized \textit{NAM} genes in developing flowers. It is shown that, after floral-boundary initiation, the \textit{NAM} gene \textit{Solyc03g115850} (\textit{SlNAM2}) is expressed as stripes that mark the boundaries between sepals and between different floral whorls. Furthermore, ectopic accumulation of \textit{SlNAM2}-encoding transcripts caused various growth-suppression and extraorgan phenotypes typically observed in plants over-expressing known boundary genes. Flower-specific silencing of sly-miR164-targeted \textit{NAM} genes (\textit{AP1}>>\textit{MIR164}) caused defects in the separation of sepals and floral whors indicating abnormal boundary specification. However, supplementing these \textit{NAM}-deficient flowers with miR164-resistant \textit{SlNAM2} suppressed their fusion phenotypes and completely restored floral boundaries. Together, our results strongly suggest that \textit{SlNAM2} participates in the establishment of tomato flower whorl and sepal boundaries.

Key words: Boundary, development, flower, miR164, NAC, tomato.

Introduction

The production of plant lateral organs depends on the formation of a narrow domain of non-dividing cells or boundary that separates the organ primordium from the meristem. In addition, normal lateral organ architecture requires proper organ–organ boundary formation that separates distinct tissues (Breuil-Broyer \textit{et al.}, 2004; Aida and Tasaka, 2006b; Rast and Simon, 2008). It was found that the formation of boundaries is regulated by specific boundary genes that are expressed in the cells that will form the boundary. Accordingly, misexpression of these genes can lead to growth arrest or abnormal development (Aida \textit{et al.}, 1997; Brewer \textit{et al.}, 2004; Takeda \textit{et al.}, 2004; Aida and Tasaka, 2006b). In \textit{Arabidopsis thaliana}, several gene families are involved in the specification of meristem–organ and organ–organ boundaries. Prominent among them is the large family of plant-specific NAM, ATAF1/2, CUC2 (NAC) transcription factors which include several genes that participate in the establishment of meristem–organ and organ–organ boundaries (Ishida \textit{et al.}, 2000; Takada \textit{et al.}, 2001; Aida and Tasaka, 2006b; Raman \textit{et al.}, 2008). A typical NAC transcription factor contains a highly conserved N-terminal DNA-binding NAC domain and a variable C-terminal region (Ooka \textit{et al.}, 2003). Alignment of the
C-terminal regions of closely related NAC proteins identifies common sequence motifs that correlate with their functionality. For example, the motifs LPPLxD and [E/x][H/x] VxCFS[N/x] have been found in most Arabidopsis and rice NAC proteins which belong to the NAM subclade and are involved in developmental plans (Ooka et al., 2003). In addition to boundary morphogenesis, NAC genes are involved in various developmental processes such as root formation (Xie et al., 2000), leaf senescence (Kim et al., 2009), and biotic and abiotic stress responses (Delessert et al., 2005; He et al., 2005).

The Arabidopsis CUP SHAPED COTYLEDON1 (CUC1) and CUC2 are functionally redundant miR164-regulated NAM genes that promote boundary formation and maintenance throughout vegetative and reproductive development (Aida et al., 1997; Takada et al., 2001). Accordingly, they are expressed between the meristem and the new lateral organ, at the base of the outgrowing teeth defining the leaf sinuses, between the inflorescence meristem and the new flower meristem, and between floral-organ primordia (Ishida et al., 2000; Takada et al., 2001; Vroemen et al., 2003; Nikovics et al., 2006). Consistent with their requirement for boundary formation, cuc1 cuc2 double mutants develop fused cotyledons and produce flowers with fused sepals and stamens and with fewer petals (Aida et al., 1997).

Similar phenotypes have also been observed in plants over-expressing miR164 (Laufs et al., 2004; Mallory et al., 2004). Gain-of-function of either CUC1 or CUC2 leads to extrafloral organ formation and variable growth-suppression phenotypes, which have been suggested to be caused by repression of cell division (Takada et al., 2001; Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007).

To date, only a single NAM gene involved in boundary formation has been functionally characterized in tomato. A loss-of-function mutant of this gene produced goblet-shaped fused cotyledons and, accordingly, it was named GOBLET (GOB). Similar to its CUC2 homologue, the spatial and temporal expression of GOB is post-transcriptionally regulated by sly-miR164. This regulation limits GOB expression to the boundaries between the shoot apical meristem and leaf primordia and between leaflet primordia (Blein et al., 2008; Berger et al., 2009). Accordingly, a loss-of-function gob-3 mutant produced smaller leaves with smooth leaflet margins lacking secondary leaflets, and gain-of-function gob-4d produced extralobed cotyledons and deeply lobed leaves, together indicating that GOB is required for the formation of the boundaries between leaflets in compound tomato leaves (Berger et al., 2009). In the flower, GOB is expressed at the boundaries between floral meristem and floral-organ primordia (Blein et al., 2008). In addition, gob-3 and gob-4d mutants produced flowers with fused sepals and fewer locules or with extra carpals, respectively, together suggesting that GOB functions in the formation of floral-organ boundaries as well (Berger et al., 2009).

In the current study, SINAM2, a new NAM gene that is post-transcriptionally regulated by sly-miR164, is functionally characterized and evidence is provided for its involvement in the establishment of floral boundaries.

Materials and methods

Plant material and growth conditions

Tomato (Solanum lycopersicum) cv. M82 lines 35S:LhG4 (Lischitz et al., 2006), API1:LhG4 (Fernandez et al., 2009), and OP:MI R164 (Alvarez et al., 2006) have been described elsewhere. The tomato plants were grown under greenhouse conditions with temperatures ranging between 15 °C and 25 °C in a tufa-peat mix with nutrients, using 41 pots. Germination and seedling growth took place in a growth chamber with a 16/8 h light/dark period (photosynthetic photon flux density: 50–70 μmol m–2 s–1) at a constant temperature of 24 °C. For crosses, closed flowers were emasculated by removing the petals and stamens and hand-pollinated with the pollen of an appropriate homzygous driver line.

Total RNA extraction and small-RNA blot analyses

Total RNA was isolated from different tomato tissues with Bio-TRI RNA reagent (Bio-Lab, Jerusalem, Israel) according to the manufacturer’s protocol. After the addition of isopropanol, the RNA extract was incubated overnight at −20 °C to enhance the precipitation of low-molecular-weight RNAs. Following an ethanol wash, RNA was resuspended in RNase-free water and kept at −80 °C until use. Small-RNA gel blot analysis of 5 μg total RNA was performed as described previously by Talmor-Neiman et al. (2006). For the detection of sly-miR164 and U6 small nuclear RNA, a radiolabelled oligo probe that is complementary to the corresponding small RNA was used. For the detection of the SINAM2 small interfering RNAs (siRNAs), a 353 bp fragment from the 3’ untranslated region (UTR) of SINAM2 was amplified by RT-PCR from tomato flower cDNA with the primers SLNAM2IR_ClaI-PstI_fwd and SINAM2IR_HindIII-EcoRI_rev (all primer sequences are given in Supplementary Table S1 at JXB online) and then cloned into pGEM-T Easy (Promega, Madison, WI, USA) so that it was in the antisense orientation relative to the T7 promoter. Then a radiolabelled RNA probe was transcribed using the RiboScribe T7 probe synthesis kit (Epicentre Biotechnologies, Madison, WI, USA) in the presence of [α-32P]UTP.

Target prediction and validation by cleavage-site mapping

Sly-miR164 targets were predicted by psRNATarget (http://plantgrn.noble.org/psRNATarget/, last accessed 09 September 13) (Dai and Zhao, 2011) against the current version of the publicly available genome [SGN ITAG release 2.3 predicted cDNA (SL2.40)]. For target validation, total RNA was extracted from tomato flowers as described above, and enriched for poly(A) mRNA using the Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA). A modified procedure for RNA ligase-mediated rapid amplification of cDNA ends (5’ RLM-RACE) was performed with the GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) as described previously by Talmor-Neiman et al. (2006). Briefly, cDNA was amplified with the GeneRacer-5’ primer and with SINAM2_RACE, SINAM3_RACE, SINAC1_RACE, and GOBLET_RACE primers followed by nested PCR using GeneRacer-5’-nested primer and SINAM2_RACE nested, SINAM3_RACE nested, SINAC1_RACE nested, and GOBLET_RACE nested primers, respectively. The PCR conditions used for both amplification steps were as recommended by the manufacturer. The amplified products were gel-purified, cloned into pGEMT-easy vector (Promega) and sequenced. For transgenic SINAM2, total RNA was extracted from young tomato leaves as described above, and 6 μg DNA-free total RNA was used to produce the RLM-RACE cDNA. The cDNA was subjected to an amplification procedure with the GeneRacer-5’ primer and the 5’ RLM-RACE primer pair GeneRacer-5’IR_ClaI-PstI_fwd and SlNAM2IR_ClaI-PstI_rev. The amplified product was gel-purified, cloned into pGEMT-easy vector (Promega), and sequenced. For transgenic SINAM2, total RNA was extracted from young tomato leaves as described above, and 6 μg DNA-free total RNA was used to produce the RLM-RACE cDNA. The cDNA was subjected to an amplification procedure with the GeneRacer-5’ primer and the 5’ RLM-RACE primer pair GeneRacer-5’IR_ClaI-PstI_fwd and SlNAM2IR_ClaI-PstI_rev.
Plasmids construction

For the SlNAM2 reporter construct, the coding region of SlNAM2 was cloned by RT-PCR from the flower cDNA with the primers XhoI-U218896_fwd and BamHI-U218896_rev, which contained XhoI and BamHI sites at their 5’ ends, respectively. The amplified fragment was restricted with XhoI/BamHI and cloned into the respective sites of the OP-TATA-BJ36 shuttle vector between an OP array (Moore et al., 1998) and Agrobacterium tumefaciens octopine synthase terminator (OCS) to generate OP:SlNAM2. To generate OP:mSlNAM2, six silent mutations in the SlNAM2 sly-miR164 target site were inserted using two-step PCR mutagenesis. Firstly, the 164-mutant-target_fwd and 164-mutant-target_rev primers were used in conjunction with BamHI-U218896_rev and XhoI-U218896_fwd, respectively, to insert six substitutions (lowercase letters in Supplementary Table S1 at JXB online) into the SlNAM2-coding region. Then, the amplified products were assembled by using them as a template for PCR with the primer pair XhoI-U218896_fwd and BamHI-U218896_rev. The amplified fragment was restricted with BamHI/XhoI and cloned into the identical sites of OP:SlNAM2, replacing the respective wild-type SlNAM2 fragment to generate OP:mSlNAM2. Following sequence validation, the NorI fragments of OP:SlNAM2 and OP:mSlNAM2 were mobilized into the pART27 binary vector (Gleave, 1992) to generate pART27-OP:SlNAM2 and pART27-OP:mSlNAM2, respectively. For the SlNAM2 RNA interference (RNAi) reporter construct, a 353-bp fragment from the 3’ UTR of SlNAM2 was cloned by RT-PCR from the flower cDNA with the primers SLNAM2R_ClaI-PstI_fwd and SlNAM2IR_HindIII-EcoRI_rev, each containing two indicated restriction sites at their 5’ end. The amplified fragment was restricted with either PstI/EcoRI or Clai/HindIII and cloned into the sense and antisense orientations, respectively, around the first intron of the Arabidopsis AKT1 gene to generate max2intpFLAP-SlNAM2IR. Following sequence validation, the XhoI fragment of the max2intpFLAP-SlNAM2IR was mobilized into the XhoI site of the OP-TATA-BJ36 shuttle vector to generate OP:SlNAM2IR. Following orientation validation, the NorI fragment of the OP:SlNAM2IR vector was mobilized into the binary vector pART27 to generate pART27-OP:SlNAM2IR.

Transformation of tomato plants

The binary vectors pART27-OP:SlNAM2, pART27-OP:mSlNAM2, and pART27-OP:SlNAM2IR were transformed into tomato cv. M82 as described previously by Stay et al. (2010). Transgenic progeny were selected by germinating sterile seeds on selective medium (1× MS medium, 3% w/v sucrose, 100 mg l–1 kanamycin), where only transgenic seedlings developed a branched root system. Further validation was performed by PCR of genomic DNA with the primer pairs OCS_rev and SlNAM2_mir164_target_fwd or SlNAM2_mmiR164_target_fwd to detect the OP:SlNAM2 and OP:mmiR164 transgenes, respectively and with the primer pair pFlap_intron_fwd and SLNAM2IR_ClaI-PstI_fwd to detect the OP:SlNAM2IR transgene.

Real-time quantitative (q) RT-PCR analyses

Total RNA was extracted from different tomato tissues as described above. Total RNA samples were treated with RNase-free DNase (Fermentas Life Sciences, Vilnius, Lithuania) to eliminate genomic DNA contamination. The concentration and integrity of the RNA samples were determined by an ND1000 spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA) and by gel analysis, respectively. First-strand cDNA was synthesized from 2 µg of total RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) following the manufacturer’s instructions. An RT-negative control was used to ensure the absence of genomic DNA template in the samples. The PCRs were performed with Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) in a Rotor-Gene 6000 cycler (Qiagen). To ensure the specificity of the amplified fragment, the amplicons were verified by sequencing. Furthermore, at the end of each PCR run, the melting temperature of the product was determined to verify the specificity of the amplified fragment. PCR products were analysed using Rotor Gene Series 6000 software version 1.7 (Qiagen). Two to three independent biological replicates were used for each sample (as indicated), and quantifications were performed in triplicate. The relative expression levels of GOB, SINAC1, SINAM2, and SINAM3 mRNA were calculated using a two-standard curve method normalized to TIP41 as a reference gene.

Histological analysis

Analysed tissues were fixed in FAA (Formalin, Acetic acid, Alcohol) until use, then dehydrated in increasing concentrations of ethanol, cleared with histoclear, and embedded in paraffin. Sections cut by microtome to 10 µm thickness were placed on microscope slides and stained with 1% (w/v) Safranin followed by 0.2% (w/v) Fast Green. Slides were examined under bright-field using a Leica light microscope equipped with a camera.

Results and discussion

Sly-miR164 guides the cleavage of four NAC-domain genes in tomato

MiR164 is a conserved, important regulator of the CUC genes which are involved in vegetative as well as reproductive organ-boundary formation (Aida et al., 1997; Laufs et al., 2004). BLASTN with mature miR164 sequences (miRBase, release 19, http://www.mirbase.org/, last accessed 09 September 13) versus our tomato deep-sequenced small RNA data set (Hendelman et al., 2013) and the publicly available tomato small RNA sequences (Tomato Functional Genomics Database) revealed two putative miR164-like sequences (data not shown), but only the two genomic loci encoding the ath-miR164a-identical ones could fold into a pre-miRNA-like hairpin structure (Fig. 1A; see Supplementary Fig. S1A at JXB online). In addition, the corresponding sly-miR164* strand encoded by each hairpin was identified in our small RNA data set, validating their functionality as sly-miR164 precursors (Fig. 1A). An additional query of the tomato genome did not identify any novel miR164 sequences suggesting that the identified sly-miR164 is the only miR164 family member encoded by the tomato genome. RNA gel blot was used to analyse the expression of sly-miR164 in vegetative and reproductive tomato tissues. This analysis indicated that sly-miR164 is most abundant in open flowers and ripen fruit (Fig. 1B). In tomato, sly-miR164 has been found to negatively regulate the CUC2-like transcription factor GOB (Berger et al., 2009). To identify additional NAC-domain genes that are subjected to
sly-miR164-guided cleavage in flowers, candidate mRNA targets were predicted and their cleavage was validated by RLM-RACE. This analysis confirmed that in addition to GOBLET (Solyc07g062840), three mRNA targets—Solyc03g115850, Solyc06g069710, Solyc07g066330—and Solyc07g066330 were guided to cleavage by sly-miR164 in tomato flowers (Fig. 1C). Sequence analysis of their putative open reading frames indicated that they encode NAC-domain proteins. In addition, this analysis revealed that they all contain the signature motifs LPPLxD and [E/x][H/x]VxCFS[N/x] in their C-terminal region, which predict the involvement of NAC-domain proteins in developmental programmes (see Supplementary Fig. S1B at JXB online) (Ooka et al., 2003). Phylogenetic reconstruction of the corresponding tomato and Arabidopsis NAC-domain proteins indicated that Solyc07g066330 (SlNAC1) encodes a homologue of Arabidopsis miR164-regulated NAC1 which has been found to mediate auxin signalling and to promote lateral root development (Xie et al., 2000; Guo et al., 2005); the related Solyc03g115850 (SINAM2) and Solyc06g069710 (SINAM3) proteins (60%/70% identity/similarity), which belong to the same group as the CUC proteins, were distanty related to ORESARA1 (ORE1) which has been found to positively regulate ageing-induced cell death in Arabidopsis leaves (Fig. 1D) (Kim et al., 2009).

**Flower-specific silencing of sly-miR164 target genes disturbs whorl and sepal separation**

To investigate the involvement of the sly-miR164-targeted NAM genes in flower-boundary formation, sly-miR164 was over-expressed in the flower primordia by transactivation of the previously characterized M82 tomato OP:MIR164 responder line with the available flower-specific AP1:LhG4 driver line, which drives expression throughout young floral primordia (Hendelman et al., 2013). First, sly-miR164 over-expression was validated by Northern analysis of young AP1>>MIR164 buds revealing a 3-fold increase in its levels compared with control buds (Fig. 2A). This increase
was consistent with the significant reduction in \textit{GOB} (70\%) and \textit{SinAC1}, \textit{SinAM2}, and \textit{SinAM3} (~95\%) accumulation in these buds, further corroborating their targeting by sly-miR164 (Fig. 2B). Phenotypic analysis of silenced \textit{API}>>\textit{MIR164} flowers revealed elongated sepals that were fused to each other at various points (Fig. 2C). Moreover, failure of these sepals to peel away from the flower suggested the occurrence of partial fusion between the first and second whorls (Fig. 2C). Indeed, transverse sectioning of young \textit{API}>>\textit{MIR164} buds at the base of the style showed that the three outer whorls and their organs were not separated at that stage whereas, in control buds, they were completely separated (Fig. 2D). Accordingly, longitudinal sectioning of fully developed \textit{API}>>\textit{MIR164} flowers showed that the three outer whorls and, in addition, the fourth whorl separated later than in controls (Fig. 2D). Together, these phenotypes indicated that sly-miR164 target genes are required for the normal formation of flower sepal and interwhorl boundaries. The \textit{gob-3} loss-of-function tomato mutant has been shown to produce flowers with increased sepal fusions and fewer locules, and to set fruit with fused outer floral organs, suggesting that \textit{GOB} is central to the formation of tomato-flower boundaries (Blein \textit{et al.}, 2008; Berger \textit{et al.}, 2009). Thus, it is highly likely that the reduced levels of \textit{GOB} in \textit{API}>>\textit{MIR164} flower primordia are responsible for at least some of the defective boundary phenotypes. Nevertheless,
since GOB silencing in AP1>>MIR164 flowers was driven by a heterologous promoter and was not complete as in the gob-3 loss-of-function mutant flowers, the AP1>>MIR164 defective flower-boundary phenotypes might be the result of the down-regulation of GOB and either one or a combination of the other sly-miR164 target genes.

**SINAM2 is expressed in floral boundaries**

Since SINAC1 probably represents a homologue of Arabidopsis NAC1 (Fig. 1D), which has not been implicated in flower-boundary formation (Guo et al., 2005), its contribution to the boundary-defective phenotype was less likely. In addition, SINAM2 was much more abundant than SINAC1 and SINAM3 in developing flowers (see Supplementary Fig. S2 at JXB online). Thus, to examine the possible involvement of SINAM2 in flower-boundary establishment, its spatial expression in young buds was determined by in situ hybridization. Wild-type M82 tomato flowers are composed of four whorls of distinct floral organs. Longitudinal and successive transverse sections of stage 8 buds showed the spatial separation of the whorls and their corresponding floral organs (Fig. 3A–E). A transverse section of a relatively proximal part of a young bud showed completely fused whorls (Fig. 3B). At that same location, stripes of SINAM2 mRNA were expressed at the boundary between the first and second whorls prior to their separation (Fig. 3F). In a more distal plane, the first and the fourth whorl are clearly separated from the second and third, respectively, but the perianth organ primordia are still laterally fused (Fig. 3C). At that position, stripes of SINAM2 mRNA were detected at the boundaries between the second and the third and the third and the fourth whorls. In addition, SINAM2 mRNA marked the lateral margins of the sepals and surrounded the stamen filaments (Fig. 3G). At a more distal plane, all whorls were separated (Fig. 3D) and SINAM2 mRNA was detected between the fused sepals (Fig. 3H). In the most distal section of the ovary, the sepals, which protect the bud, were the only organs that remained fused to each other (Fig. 3E) and that fusion was marked by SINAM2 mRNA (Fig. 3I). In accordance with its strong sly-miR164-mediated silencing in AP1>>MIR164 buds (Fig. 2B), SINAM2 expression was not detected in them, further confirming the authenticity of the wild-type in situ signal (Fig. 3J–M). Taken together, the SINAM2 transcript was expressed at the boundaries between adjacent sepals and whorls suggesting that it might be involved in their separation. However, no significant SINAM2 mRNA signal could be detected before the fusion of carpels (bud stages 1–6; data not shown), indicating that it is poorly expressed at the time of whorl-boundary formation. GOB is expressed in the boundaries between the floral meristem and floral-organ primordia (Blein et al., 2008). This implicates GOB rather than SINAM2 in whorl boundary formation and raises the possibility that SINAM2 is involved in floral-boundary maintenance.

**Accumulation of SINAM2-encoding transcript is associated with growth-repression phenotypes**

Cell proliferation is greatly reduced in the organ–meristem and organ–organ boundaries (Breuil-Broyer et al., 2004). This process is mediated by the activity of regulatory boundary genes (Aida and Tasaka, 2006a) and plays a role in organ morphogenesis (Nikovics et al., 2006). While loss-of-function mutations in these genes result in overgrowth of the boundary region, manifested as organ fusions, over-accumulation of these genes due to gain-of-function or ectopic expression usually represses growth, manifested as smaller and occasionally multiple organs.

**Fig. 3.** Expression pattern of SINAM2 in tomato bud. (A) Longitudinal section of wild-type flower buds at stage 8. The lines mark the positions of the cross-sections shown in (B)–(E). Arrowheads mark the points of separation between corresponding whorls. (B–M) Successive transverse sections from the base upward. (B–E) Safranin-Fast Green differential staining. (F–M) In situ hybridization with SINAM2 antisense probe in the indicated genotypes. Arrowheads and arrows mark SINAM2 signal between whorls and around floral organs, respectively. S. sepal; P, petal; A, anther; O, ovary. Scale bars=100 µm.
Fig. 4. Phenotypic and molecular characterization of 35S>>SlNAM2 and 35S>>mSlNAM2 plants. (A–C) Phenotypes of isolated cotyledons 11 d after sowing from control (35S:LhG4) and transactivated (35S>>SlNAM2 and 35S>>mSlNAM2) tomato seedlings. Multiple cotyledons were separated. (D) Four-month-old plants of the indicated genotypes. (E) Representative flower at anthesis of the indicated genotypes. (F) Stamen and pistil phenotypes of the indicated genotypes. (G) Transverse sections of the ovary of the indicated genotypes. (H) Style lengths of the indicated genotypes. Data are means ±SD (n=10). Different letters indicate statistically significant difference as determined by Student’s t test (P ≤0.01). (I) QRT-PCR analysis of SlNAM2-encoding transcripts in the flowers of indicated genotypes. Primers were designed around the corresponding sly-miR164 complementary site. TIP41 expression values were used for normalization. Data are means ±SD of two biological replicates, each measured in triplicate. Different letters indicate statistically significant difference as determined by Student’s t test (P ≤0.01). Scale bars: (A–C)=1 cm; (D)=10 cm; (E, F)=5 mm; (G)=500 µm.
and extra and elaborate lobing of cotyledons, leaves, and floral organs (Hiratsu et al., 2002; Brewer et al., 2004; Baker et al., 2005; Berger et al., 2009; Busch et al., 2011; Huang et al., 2012).

Since SINA2 is expressed at flower boundaries, the question was asked whether it has similar boundary gene activity and can suppress growth when accumulated. To investigate this, two homozygous tomato responder lines were generated that are able to express wild-type (SINA2) and sly-miR164-resistant (mSINA2) versions of the gene upon transactivation (for further details see the Material and methods and see Supplementary Fig. S3 at JXB online). Both were crossed with the strong constitutive 35S:LhG4 and flower-specific AP1:LhG4 driver lines to generate corresponding transactivated F1 progeny plants. Inactivated OP:SINA2 and OP:mSINA2 responder plants were morphologically identical to the driver lines and wild-type M82 tomato (data not shown). However, 35S>mSINA2 plants and, to a much lesser extent, 35S>SINA2 plants showed various growth-repression-associated phenotypes. Compared with control tomato cotyledons which are oval and entire, the 35S>mSINA2 cotyledons were abnormally shaped, smaller and lobed, and occasionally three instead of two cotyledons were produced (Fig. 4A–C). A similar but less pronounced phenotype was observed in 35S>SINA2 cotyledons, which were larger than 35S>mSINA2 (Fig. 4B). Reminiscent multiple and serrated cotyledon phenotypes have also been reported as a result of expression of the GOB sly-miR164-resistant mutant gene Gob-4d under its native or leaf-specific FIL promoter, respectively (Berger et al., 2009). In addition, mature 35S>mSINA2 plants were dwarf whereas the 35S>SINA2 plants were no different from the controls (Fig. 4D). Moreover, examination of 35S>mSINA2 flowers revealed a reduction in flower size and wrinkled and slightly lobed petals (Fig. 4E). Also, compared with the control and 35S>SINA2, dramatic growth repression was observed in the two inner whorls of 35S>mSINA2 flowers, including shorter stamens and style (Fig. 4F, H). Moreover, the pistil was very wide as a result of extra carpel formation (Fig. 4G). QRT-PCR analysis of control and transgenic flowers revealed the relatively mild accumulation of SINA2 in 35S>SINA2 compared with the controls and, consistent with mSINA2 resistance to

![Fig. 5](image-url)  

**Fig. 5.** Phenotypic characterization of AP1>>SINA2 and AP1>>mSINA2 flowers. (A) Representative flower at anthesis of the indicated genotypes. The upper panel presents the whole flower and the lower panel presents the anthers and pistil after the sepals and petals were removed. Scale bars=1 mm. (B) Sepal areas of the indicated genotypes. Data are means ±SD (n ≥70). Different letters indicate statistically significant difference as determined by Student’s t test (P≤0.01). (C) Style lengths of the indicated genotypes. Data are means SD (n ≥10). Letters indicate statistically significant differences as determined by Student’s t test (P 0.01).
sly-miR164 cleavage, a much higher accumulation of SINAM2-encoding transcript was detected in 35S>>mSlNAM2 flowers (Fig. 4I).

Similarly, high accumulation of SINAM2-encoding transcript in AP1>>mSlNAM2 buds (see Supplementary Fig. S4 at JXB online) was associated with significantly smaller sepals and styles and slightly lobed petals compared with the organs of control OP:SINAM2 flowers (Fig. 5), whereas less accumulation in AP1>>SlNAM2 buds resulted in milder organ phenotypes (Fig. 5). Together, these results demonstrated a positive correlation between the accumulation levels of SINAM2-encoding transcript and abnormalities typically observed in plants over-expressing boundary genes, suggesting similar activity for SlNAM2.

**SlNAM2 accumulation rescues the fusion phenotypes of AP1>>MIR164 flowers**

Our results indicated that SINAM2 is expressed at floral whorl and organ boundaries and might suppress growth

**Fig. 6.** Restoration of normal flower phenotype upon expression of mSlNAM2 in miR164-over-expressing flowers. (A) Flower phenotypes of the indicated genotypes. The upper panel presents the whole flower; the middle panel presents a whole flower on its side, and the lower panel presents isolated anthers and pistil. Arrowheads mark the points of sepal fusion. Scale bars=1 mm. (B) Sepal and fusion lengths of the indicated genotypes. (C) Longitudinal section of flowers before anthesis from the indicated genotypes. Scale bars=100 µm. S, sepal; P, petal; A, anther; O, ovary; St, style. (D) Quantitative RT-PCR analysis of GOBLET, SlNAC1, SlNAM2, and SlNAM3 in 1–2 mm buds of the indicated genotypes. Primers were designed around the corresponding miRNAs complementary site. TIP41 expression values were used for normalization. Data are means ±SD of three biological replicates, each measured in triplicate.
when accumulated. In the absence of an informative loss-of-function mutant (see Supplementary Fig. S5 at JXB online), the question was then asked whether SINAM2 growth suppression activity can define floral boundaries. To that end, $AP1\text{>>MIR164}$ mutant flowers, which had fused sepals and abnormal whorl separation, were complemented with SINAM2 and the resulting phenotype was analysed. This was done by expressing $m\text{SINAM2}$ in the background of $AP1\text{>>MIR164}$ plants ($AP1\text{>>MIR164} \rightarrow m\text{SINAM2}$). As a control, SINAM2 was expressed on the same genetic background ($AP1\text{>>MIR164} \rightarrow \text{SINAM2}$). As expected, analysis of $AP1\text{>>MIR164} \rightarrow \text{SINAM2}$ flowers showed elongated fused sepals and abnormal interwhorl fusion (Fig. 6A–C). This abnormal phenotype was different from that of $AP1\text{>>MIR164}$ flowers (Fig. 2C, D). By contrast, the $AP1\text{>>MIR164} \rightarrow m\text{SINAM2}$ flowers had a wild-type-like phenotype. Although their sepals were slightly shorter than controls they were not fused (Fig. 6A, B) and contained no abnormal interwhorls (Fig. 6A, C). QRT-PCR of young buds revealed accumulation of the sly-miR164-resistant $m\text{SINAM2}$ in $AP1\text{>>MIR164} \rightarrow m\text{SINAM2}$ whereas the sly-miR164-sensitive SINAM2, SINAC1, SINAM3, and GOB were silenced (Fig. 6D). These results demonstrated that, when precociously expressed, SINAM2 is able to restore the formation of floral boundaries.

In Arabidopsis leaves, complementation of the cuc2 mutant by a NAC-domain gene did not occur unless they had redundant functions. NAC1, ANAC019, and to some extent, CUC3, which have different functionalities from that of CUC2, were not able to restore leaf morphogenesis whereas the redundant gene, CUC1, could (Hasson et al., 2011). Thus, the boundary restoration ability of SINAM2 might reflect some functional redundancy with GOB, which was suggested to be required for the formation of tomato flower boundaries (Blein et al., 2008). Still, the relative over-accumulation of SINAM2-encoding transcripts needed for boundary restoration and the milder phenotypes of $35S\text{>>SINAM2}$ compared with $35S\text{>>Gob-4d}$ plants (Berger et al., 2009), suggest that SINAM2 activities are less suitable for boundary formation than GOB.

Arabidopsis leaf serration occurs in two different phases: an early phase, requiring CUC2, during which the boundaries which surround the emerging tooth are initiated, and a later phase, requiring both CUC2 and CUC3, which maintains the boundaries to sustain teeth formation (Hasson et al., 2011). In accordance with that, CUC2 is already expressed in the leaf primordium margins before teeth outgrowth whereas CUC3 can hardly be detected at that stage and, afterwards, both are detected in the sinuses of the developing serrations (Nikovics et al., 2006; Hasson et al., 2011). In a reminiscent way, the non-overlapping expression patterns of GOB (Blein et al., 2008) and SINAM2 in floral boundaries (Fig. 3), where GOB precedes SINAM2 expression, may suggest that they function at different stages of boundary morphogenesis. The occurrence of SINAM2 in the floral-boundary after they were initiated by GOB might imply its function in boundary maintenance rather than in their formation. However, to support this hypothesis, the analysis of the SINAM2 loss-of-function mutant is required.

### Supplementary data

Supplementary data can be found at JXB online.

**Supplementary Table S1.** Primers used in this study.

**Supplementary Fig. S1.** Sequence alignment of sly-miR164-targeted NAC transcription factors.

**Supplementary Fig. S2.** Quantitative RT-PCR analysis of sly-miR164-targeted genes in developing flowers.

**Supplementary Fig. S3.** Generation of $OP:\text{SINAM2}$ and $OP:m\text{SINAM2}$ responder lines.

**Supplementary Fig. S4.** Quantitative RT-PCR analysis of SINAM2 in stage 9 buds.

**Supplementary Fig. S5.** Molecular analysis of $35S\text{>>SINAM2IR}$ plants.

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