The WUSCHEL-related homeobox1 gene of cucumber regulates reproductive organ development

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Abstract

The WUSCHEL-related homeobox1 (WOX1) transcription factor plays an important role in lateral growth of plant organs; however, the underlying mechanisms in the regulation of reproductive development are largely unknown. Cucumber (Cucumis sativus) has separate male and female flowers, facilitating the study of the role of WOX1 in stamen and carpel development. Here, we identified a mango fruit (mf) mutant in cucumber, which displayed multiple defects in flower growth as well as male and female sterility. Map-based cloning showed that Mf encodes a WOX1-type transcriptional regulator (CsWOX1), and that the mf mutant encodes a truncated protein lacking the conserved WUS box. Further analysis showed that elevated expression of CsWOX1 was responsible for the mutant phenotype in cucumber and Arabidopsis. Comparative transcriptome profiling revealed certain key players and CsWOX1-associated networks that regulate reproductive development. CsWOX1 directly interacts with cucumber SPOROCYTELESS (CsSPL), and many genes in the CsSPL-mediated pathway were down-regulated in plants with the mutant allele at the Mf locus. In addition, auxin distribution was affected in both male and female flowers of the mutant. Taking together, these data suggest that CsWOX1 may regulate early reproductive organ development and be involved in sporogenesis via the CsSPL-mediated pathway and/or modulate auxin signaling in cucumber.

Keywords: Cucumber (Cucumis sativus L.), flower development, mango fruit mutant, NZZ/SPL, sporogenesis, WOX1.

Introduction

In higher plants, the process of leaf and flower development, from initiation to patterning, involves coordinated regulation among transcription factors, small RNAs and hormones (Moon and Hake, 2011). Among the many genes and associated networks identified, the plant-specific WUSCHEL-RELATED HOMEobox (WOX) transcription factors serve as master switches controlling key developmental programs (Costanzo et al., 2014). In Arabidopsis, the WOX gene family consists of 15 members including the founder member WUSCHEL (WUS) and WOX1–WOX14 (Haecker et al., 2004). WOX genes can be classified into three clades: the modern WUS clade (WUS and WOX1–7), the intermediate clade (WOX8, 9, 11, and 12), and
the ancient clade (WOX10, 13, and 14) (van der Graaff et al., 2009). Most WUS clade members are involved in regulating stem cell homeostasis. For example, WUS is required to maintain the stem cell population in the shoot (Lau et al., 1996; Mayer et al., 1998). WOX1 and WOX3/PRS (PRESSED FLOWER) seem to function redundantly to coordinate adaxial/abaxial patterning through interaction with adaxial and abaxial domain-specific polarity factors (Matsumoto and Okada, 2001; Vandenbussche et al., 2009; Nakata et al., 2012). WOX3/PRS is required for the development of lateral sepals and stamens in the flower and of the stipules at the leaf base (Shimizu et al., 2009). The maize (Zea mays) NS1 (for narrow sheath) and NS2 belong to the PRS/ WOX3 subfamily; the ns1ns2 double mutant displays a reduced leaf blade (Scanlon et al., 1996; Nardmann et al., 2004).

Mutants of Arabidopsis WOX1 gene homologs have been identified in several other plant species, including bladeless (lam1) in Nicotiana sylvestris (McHale and Marcotrigiano, 1998), maewest (mau) in Petunia × hybrida (Vandenbussche et al., 2009), stenofolia (stf) in Medicago truncatula (Taddege et al., 2011), and lathyroides (lath) in pea (Pisum sativum) (Zhuang et al., 2012). A common feature of these mutants is narrower leaves; however, the leaf length is virtually unaffected. In the mau or stf mutant, the petals are narrower or fail to fuse; in the lath mutant, the lateral growth of stipules, leaflets, and petals is reduced, with the tendril and dorsal petal being the most affected. Investigation of these mutants supports the conserved and critical function of WOX1 in lateral growth of leaf and flower organs. Nevertheless, studies of WOX1 function have so far been limited to a small number of species and have focused mainly on its role in leaf growth or flower organ development separately. All reported mutants show varying degrees of male or female fertility; however, the direct link between WOX1 gene functions and male or female fertility is unclear.

Cucumber (Cucumis sativus L., 2n=2x=14) is an important vegetable worldwide, belonging to the Cucurbitaceae family, which also includes several other important cucurbit crops such as melon (C. melo L.), watermelon (Citrullus lanatus), and pumpkin/squash (Cucurbita spp.). Unlike other species, stamens and carpels are usually developed in separate flowers, producing unisexual male and female flowers, respectively, in the majority of cucurbit plants. Therefore, the functions of WOX in separate unisexual flowers in this family should be interesting. Nevertheless, the WOX gene family members have not been investigated in any cucurbit species. In the present study, we identified a spontaneous cucumber mutant, mango fruit (mf), which exhibited extensive morphological differences in leaves, flowers, fruit, and seeds. We found that the mutant phenotype was caused by a 1 bp deletion in the coding region of the CsWOX1 gene, a homolog of Arabidopsis WOX1. Therefore, we characterized CsWOX1, focusing on its function in regulating flower development in cucumber.

Materials and methods

Plant materials and phenotypic characterization

The mango fruit (mf) mutant line was a spontaneous mutant identified from the cucumber line ‘Extra Early Majestic’ (AM218). In a 2014 field trial, a selfing population of the inbred line AM218 was found to be segregating at the mf locus in a 3 to 1 ratio, suggesting this is an F2 population. In this study, the wild-type (WT) and mutant plants were designated as AM218W and AM218M, respectively, and this first F2 population was designated as AM218F2. In addition, plants from open-pollinated (OP) seeds from the mutant plants in the field (F1 from OP) were self-pollinated to develop a second F2 population (AM218OPF2) for primary mapping using simple sequence repeat (SSR) markers. Then, SSR markers flanking the mf locus were used to identify plants that were heterozygous (AM218H, Mmf) at the mf locus in AM218F2, and one such individual was crossed with cucumber line 9930 to develop a third large population (AM218-9930F2) for fine mapping. Only the plants with the mutant phenotype (e.g. wrinkled cotyledons, striped true leaves) in this F2 population were employed for map-based cloning. All populations were grown in the Walnut Street Greenhouses of the University of Wisconsin at Madison. The Arabidopsis ecotype Columbia (Col-0) was used for transgenic investigation. All Arabidopsis plants were grown in a growth chamber under 16 h light/8 h dark at 22 °C.

Microscopic phenotypic characterization of the male and female flower buds from WT and mutant cucumber plants and Arabidopsis flowers were performed using a stereomicroscope (S8APO, Leica, Germany). Shoot tips and floral buds at various stages were fixed, embedded, sectioned, and dewaxed following the method of Bai et al. (2004). The sections were then observed, measured and photographed under a light microscope. For scanning electron microscopy (SEM), samples of Arabidopsis flowers and pollen were fixed, dried, dissected, and coated following the method of Bai et al. (2004), and then examined under a scanning electron microscope (JSM-6360, JEOL, Japan).

Map-based cloning of the mf locus and candidate gene sequence analysis

Initial mapping of the mf gene was performed concurrently in the AM218F2 and AM218OPF2 populations using bulked segregant analysis. In the AM218OPF2 population, two bulks, each containing an equal amount of genomic DNA from 10 WT and 10 mutant plants, were pooled and screened using SSR markers. In the AM218F2 population, DNA from 28 WT and 28 mutant plants was pooled to construct two bulks that were subjected to whole genome resequencing using the Illumina GAIIx Sequencer (Bulk-Seq). High quality reads were aligned to the Gy14 draft genome (V1.0) (Yang et al., 2012) using the BWA software (Li and Durbin, 2009). Single nucleotide polymorphism (SNP) calling was performed using SAM tool software (Li and Durbin, 2009). The SNP index and A(SNP index) were calculated to identify candidate region(s) of the mf gene (Abe et al., 2012). Average SNP indexes of SNPs located in a given genomic interval were calculated using sliding window analysis with a 2 Mb window and 10 kb increments along the Gy14 reference genome. For fine mapping, new markers in this interval were developed and applied in the AM218-9930F2 population following the method of Li et al. (2011) to delimit the mf gene in the final interval. The whole genome sequence of the interval was cloned and sequenced in both the WT and mutant cucumber lines. The candidate gene(s) were first annotated with the Cucurbits Genomics Database (http://cucurbitgenomics.org/), and then identified by searching the coding sequence (CDS) databases in Cucurbits Genomics Database and NCBI (https://blast.ncbi.nlm.nih.gov/). DNAMAN v6.0 software (http://dnaman sofware.informer.com/6.0/) was used to compare and align DNA and the deduced protein sequences between the two lines. All primers for the markers are listed in Supplementary Table S1 at JXB online.

Genome-wide identification and phylogenetic analysis of Arabidopsis WOX gene homologs in the cucumber genome

The Hidden Markov Model (HMM) profile of the HD domain (PF00046) was downloaded from the Protein family (Pfam) database (http://pfam.xfam.org/) and used to search the cucumber draft genome database (http://cucurbitgenomics.org/). All output results were searched against the NCBI database, and false-positive hits were removed. The conserved HD domain in the well-studied WOX gene family was used as a reference to filter the candidate CsWOX genes. Amino acid sequences
of the HD domain from Arabidopsis (Haecker et al., 2004) and cucumber WOX genes were aligned using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalo/). An unrooted neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 5.10 software with 1000 bootstrap replications, pair-wise deletion and a Poisson model. The sequence logo of the HD domain and WUS box were obtained using Weblogo (http://weblogo.berkeley.edu) for conserved sequences.

Quantitative real-time PCR and semi-quantitative reverse transcription PCR analysis

Shoots, female flowers (with ovaries), male flowers, leaves, and roots from WT and mutant cucumber plants were harvested. Leaves from overexpressing Arabidopsis plants were also harvested for RNA extraction. Total RNA isolation, first-strand cDNA synthesis, quantitative real-time PCR (qPCR) and reverse transcription (RT) PCR were performed following the method of Li et al. (2012). CsACTIN2 was used to normalize the expression data. For RT-PCR in Arabidopsis, the ACTIN7 gene was used as an internal reference in the expression analysis. There were three biological and three technical replicates for each gene. The relative expression was calculated according to the comparative cycle threshold (CT) method (Schmittgen and Livak, 2008). All gene-specific primers are listed in Supplementary Table S1.

Transcriptome profiling of WT and mutant cucumber lines using RNA-Seq

According to the description of Bai et al. (2004), male flower buds from stage 6 to stage 9 (observable length >0.5 and <1 mm) of AM218W and AM218M plants were employed for RNA-Seq analysis. Samples from the same plant were pooled as one biological replicate, and there were three biological replications for each genotype. RNA-Seq libraries were constructed using the NEBNext Ultra Directional RNA Library Prep kit (NEB) following the manufacturer’s instructions, and were sequenced on an Illumina HiSeq 2000 machine. Genes with at least a 2-fold change in expression between the WT and mutant lines and a false discover rate (FDR) of less than 0.05 were considered as differentially expressed genes (DEGs). Gene expression patterns of 20 selected DEGs from RNA-Seq, which were chosen for their possible roles in sporogenesis, transcriptional regulation, or hormone signaling, were verified by qPCR.

Prokaryotic expression and subcellular localization

The coding region of the WT CsWOX1 and mutant Cswox1 alleles were amplified using a Long Distance PCR Kit (Takara), and the results were confirmed by sequencing. Both gene sequences were subcloned into the pGEX-6P-1 vector using a NovoRec® PCR Kit (Novoprotein). Expression of the recombinant protein in *Escherichia coli* BL21 (DE3) and SDS-PAGE were performed as before (Tan et al., 2015). Full-length CDS of each gene was inserted into the pBI221 vector resulting in in-frame fusion with green fluorescent protein (GFP) at the N-terminus. The two recombinant plasmids were transiently transformed into Arabidopsis protoplasts (Yoo et al., 2007), and bombarded into onion epidermal cells using a PDS-1000/He Particle Delivery System (Bio-Rad, USA) following the manufacturer’s instructions. Fluorescence representing GFP expression was analysed using a confocal microscope (FV10-ASW, Olympus, Japan).

RNA in situ hybridization

The shoot apices and male and female flower buds (with ovaries) were fixed in 3.7% formalin–acetic acid–alcohol (FAA), and in situ hybridization was performed following the method of Zhang et al. (2013). Sense and antisense probes were synthesized by PCR amplification using SP6 and T7 polymerase, respectively. Probes for CsWOX1 were designed in the 5′-region of the CDS, which coexisted in both the WT and mutant genes. Primers for probe generation are listed in Supplementary Table S1.

Yeast two-hybrid and bimolecular fluorescence complementation assays

The full-length CDSs of CsWOX1, Cswox1, CsSPL (encoding cucumber SPOROCYTLESS), CsTPL (encoding cucumber transducin family protein/WD-40 repeat family protein), and CsTCP23 (encoding cucumber teosinte branched1/cyclodex/proliferating cell factor 23) were cloned into pGADT7 bait and pGBK7 prey vectors. All constructs were verified by sequencing and then transformed into the yeast strain AH109. The yeast two-hybrid (Y2H) assay was conducted according to the method of Ding et al. (2015), and 10 mM 3-amino-1,2,4-triazole (3-AT) was used to inhibit the self-activation of CsSPL in yeast cells. The combination of HAN-AD and HAN-BD was used as positive control (Zhang et al., 2013). For bimolecular fluorescence complementation (BiFC) analysis, full-length CDSs without stop codons of CsWOX1, CsSPL, CsTPL, and CsTCP23 were PCR amplified and ligated into pSPYNE-35S and pSPYCE-35S vectors containing each half of yellow fluorescent protein (YFP; N- or C-terminus) to recombine the fusion proteins in-frame. All constructs were verified by sequencing, transformed into *Agrobacterium tumefaciens* strain GV3101, and then co-transformed into the abaxial sides of 4- to 6-week-old *Nicotiana benthamiana* leaves (Ding et al., 2015). The tobacco leaves were visualized using a confocal laser microscope (LSM 510 Meta, Zeiss, Germany). The combination of IND-YFPC and SPT-YFPN was used as the positive control (Girn et al., 2011). Fluorescence signals were imaged under 488 nm excitation wavelength.

Indole-3-acetic acid (IAA) immunolocalization analysis

Cucumber male and female flower buds were soaked in 3% N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC; Sigma–Aldrich), vacuum infiltrated, incubated, and transferred into a fixation solution as previously described (Zhao et al., 2017). Samples were embedded in paraffin wax and cut into sections. After blocking in 1× phosphate-buffered saline (PBS) containing 3% (w/v) bovine serum albumin overnight (at 4°C), the sections were incubated with primary antibodies (anti-auxin antibody, mouse monoclonal; Sigma–Aldrich). Secondary antibodies (Dylight™ 488-labeled antibody to mouse IgG; Sigma–Aldrich) were diluted at 1:500 in 1× PBS and incubated for 4 h at room temperature. Negative controls of indole-3-acetic acid (IAA) immunolocalization analysis were performed on samples hybridized with the secondary antibody only. Images were obtained under a microscope (Axio, Zeiss, Germany).

Ectopic expression of CsWOX1 alleles in Arabidopsis

Full-length WT and mutant CDSs were amplified and cloned into the pBI121 vector. The recombinant plasmids were then introduced into *Agrobacterium tumefaciens* A218M plants using the floral dip method (Clough and Bent, 1998). Transgenic plants were screened on Murashige and Skoog medium with 40 mg l⁻¹ kanamycin (Sigma–Aldrich), and identified using PCR.

Accession number

The mutant Cswox1 gene sequence has been deposited to GenBank under the accession no. KY006660.

Results

Mango fruit mutant exhibits defective lateral growth of vegetative and reproductive organs

The mf mutant (AM218M) was identified in field plots (Fig. 1A1, B1). WT (AM218W) seedlings had flat, smooth, and light-green cotyledons (Fig. 1A2), which, in the mf mutant, were narrower, wrinkled, cupped downwards, and dark-green (Fig. 1B2). The mf mutant showed a significant reduction of
blade expansion of true leaves (Fig. 1A3, B3) and flower petal width (Fig. 1A4, A5, B4, B5). WT flowers had five petals that were fused at the base of the corolla (Fig. 1A4, A5), and the three carpels were clearly separated on the stigma of female flowers (Fig. 1A6). In the mutant, the five petals were narrower and more separated, with deeper incisions between the petals (Fig. 1B4, B5), and the separation of the three carpels was obscured (Fig. 1B6).

Under greenhouse conditions, the mutant was both male and female sterile, and no fruits were obtained from either self- or cross-pollinations. However, in the field, few fruits were set on the mutant plants from open pollination. WT plants set fruits that were cylindrical with more-or-less round shaped stem at flower ends (Fig. 1A7). In contrast, fruits on the mutant plant were smaller with limited growth at the flower end and were morphologically similar to a mango fruit (Fig. 1B7). Hence, this mutant was named ‘mango fruit’ (mf). The seed cavity of the mutant fruit was not developed, and the three carpels were difficult to recognize (Fig. 1A7, B7). Most fruits from the mutant plant had no seeds or deformed seeds with no embryos inside. Interestingly, among the fruits set on the mf plants, only one had a normal fruit shape, and a few (<10) ‘normal’ seeds were found inside (Fig. 1A8, B8).

Bai et al. (2004) divided the flower development in cucumber from floral meristem initiation to anthesis into 12 stages. No obvious differences were observed between WT and mf mutant before stage 6 in the developing male (Fig. 2A) and female (Fig. 2B) flower buds. From stages 7 to 9 of the male flower buds, in the WT, the anther primordia were enlarged and visually distinguished from the filament primordia, and the microsporecyte initiated, whereas in the mutant, although development of the vascular bundle in the filament primordium appeared normal, anther growth was significantly defective (Fig. 2A, stages 8 and 9). Inside the female flower bud, differences in stigma and

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**Fig. 1.** Phenotypic characterization of cucumber mango fruit mutant by comparative analysis between AM218W wild-type (A) and AM218M mutant (B) at different growth and development stages. Obvious differences between the WT and mutant lines were observed at the stage of the adult plant (A1, B1), cotyledon (A2, B2) and seedling (A3, B3), as well as in the male flowers (A4, B4), female flowers (A5, B5), stigmas (A6, B6), fruits (A7, B7), and seeds (A8, B8). All images (except A2, B2, A3, B3) were taken from plants in the field; (A6, B6) the stigma of female flowers (inset: top view). The mango-shaped fruits on mutant plants (B7) were harvested from open pollinations facilitated by bees. (This figure is available in color at JXB online.)
CsWOX1 plays key roles in cucumber flower growth

A

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<th>Stage 4</th>
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<th>Stage 7</th>
<th>Stage 8</th>
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Male Flower

B

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<th>Stage 8-3</th>
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Female Flower

Fig. 2. Comparative analysis of microscopic structure of developmental male and female flowers between the wild-type (AM218W) and mf mutant (AM218M). Floral development stage is according to Bai et al. (2004). (A) In the male flower, differences between WT and mutant are visible from stage 7. In AM218M, the vascular bundles in the filaments appear to develop normally. (B) In the female flower, the ovules begin to develop at stage 8-3 and are clearly visible at stage 9 (arrows). In the mutant, there is obviously enlarged cavity space (stage 8-2) and no ovule development (stage 9). Bars: 50 μm (A, stage 4), 80 μm (A, stages 5, 6), 100 μm (A, stages 7, 8; B, stage 6), 150 μm (B, stages 7, 8-1), 200 μm (B, stages 8-2, 8-3, 9), and 250 μm (A, stage 9). (This figure is available in color at JXB online.)

style development seemed trivial between WT and the mutant until stage 8-2 (Fig. 2B), when the mutant started showing an abnormally large cavity space. In the mutant, at stage 8-3, no ovule primordia were initiated, which resulted in a lack of an ovule structure at stage 9 (Fig. 2B).

Cucumber mango fruit encodes the transcription factor CsWOX1

Genetic analyses in the AM218F2 and AM218OPF2 populations were consistent with a single recessive gene underlying the mango fruit mutation (Table 1). Bulk-Seq was performed in the AM218F2 population, and SNP-index analysis of the sequencing data placed the mf locus in a ~2.0 Mbp interval on chromosome 1 (Fig. 3A). At the same time, linkage analysis was performed in the AM218OPF2 population, and three SSR markers (UW027083, UW027099, and UW026985) were mapped to the mf locus in a region that was consistent with the interval defined by Bulk-Seq. Thus, additional polymorphic markers were developed in this region, and the mf locus was mapped to a 0.9 cM region flanked by UW026985 and UW027099, which resided in scaffold00999 of the Gy14 genome assembly (Fig. 3B, C). A total of 894 mutant plants in the AM218-9930F2 population were used for fine mapping. Several cycles of marker development and linkage analysis with recombinants in the target region resulted in two SSR markers, UW026993 and UW027011, that delimited the mf locus into an interval of 16614 bp (Fig. 3D). The genomic DNA of this interval was cloned, sequenced, and assembled from the WT and mutant lines. In the genomic sequence, only one gene, Csa1G042780 (Fig. 3E), was annotated in the Cucurbit Genomics Database. Similarly, only one coding region was identified by searching the CDS database from Cucurbit Genomics Database and NCBI (Supplementary Fig. S1). The gene was predicted to encode a homolog of the Arabidopsis WOX1 gene; therefore, Csa1G042780 was designated as CsWOX1.

CsWOX1 has four exons (Fig. 3E; Supplementary Fig. S2) with a CDS of 1173 bp, and the deduced protein has 390 amino acid residues (Fig. 4A). CsWOX1 contains a signature homeodomain (HD) domain of the WOX family proteins and a WUS box motif (Fig. 4A). Sequence alignment between WT and the mutant revealed a 1 bp deletion in the fourth exon of CsWOX1 in the mutant (Fig. 3E; Supplementary Fig. S2), which caused a frame shift that would result in a truncated protein with 113 fewer amino acid residues in the mutant (Fig. 4A). This was confirmed by prokaryotic expression in E. coli (Fig. 4B). The mutant allele of the Mf locus was thus designated as Cswox1.
CsWOX1 is a member of WUS clade genes in cucumber

There are 11 WOX members identified in the cucumber draft genome (Supplementary Table S2). Consistent with the phylogeny of Arabidopsis WOX proteins (Haecker et al., 2004), all 11 CsWOX members could be grouped into three clades: the WUS/modern clade, the intermediate clade, and the ancient clade, with seven, two, and two members, respectively (Supplementary Fig. S3A). Based on their homology with corresponding Arabidopsis WOX proteins, the 11 cucumber genes were designated CsWOX1 to CsWOX10, as well as CsWUS.

Table 1. Segregation of the mutant mf phenotype in F2 populations derived from various cucumber backgrounds

<table>
<thead>
<tr>
<th>Population</th>
<th>Total no. of plants</th>
<th>No. of WT plants</th>
<th>No. of mutant plants</th>
<th>Expected WT to mutant ratio</th>
<th>χ²</th>
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<td>355</td>
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<td>28</td>
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<td>0.178</td>
</tr>
<tr>
<td>AM218-9930F2</td>
<td>—</td>
<td>—</td>
<td>894</td>
<td>—</td>
<td>—</td>
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</table>

For the detailed strategies used to develop the populations, see ‘Materials and methods’.

Fig. 3. Map-based cloning of the mango fruit (mf) locus. (A) From Bulk-Seq, a Δ(SNP index) graph was generated, from which the mf locus is placed in a 2 Mbp interval on chromosome 1. (B) The map location was confirmed using SSR-based linkage analysis in a small population (AM218OPF2). (C) From this 2 Mbp region, additional markers were developed in scaffold00999. (D) Fine mapping in a larger population (AM218-9930F2) delimited the mf locus into a 16 614 bp region that harbors only one predicted gene. (E) A 1 bp deletion in the fourth exon of this candidate gene (Csa1G042780) was found to be the only nucleotide variation between the WT and the mutant in this gene. ‘X’ in (D) indicates the number of recombinant events. (This figure is available in color at JXB online.)
Fig. 4. Comparison of the structure and expression of CsWOX1 candidate gene in AM218W (WT, CsWOX1) and AM218M (mutant, Cswox1). (A) Alignment of deduced amino acid residues revealed the loss of the WUS box motif in CsWOX1 protein in AM218M was caused by a 1 bp deletion whereas the homeodomain (HD) is intact. (B) Prokaryotic expression confirmed the truncated protein encoded by mutant allele Cswox1. Glutathione S-transferase (GST)-tagged CsWOX1 and Cswox1 constructs (arrows) were expressed in E. coli. IPTG− and IPTG+ are cells untreated and incubated with isopropyl-D-thiogalactopyranoside, respectively. (C) Quantitative PCR expression analysis of CsWOX1 in different tissues and organs of AM218W and AM218M plants. Expression in the roots was not detected. The expression of CsWOX1 in the mutant is significantly higher than that in the WT in all four organs and tissues. (D) Semi-quantitative RT-PCR assay of expression level of CsWOX1/Cswox1 with gene-specific and universal primers in the leaves of homozygous AM218W, AM218M, and heterozygous AM218H plants revealed basal transcription of wild-type CsWOX1 was necessary for the normal phenotype. (E) qPCR analysis of the expression level of CsWOX1 in the leaves of AM218W, AM218M, and AM218H plants revealed total mRNA accumulation of CsWOX1/Cswox1 was non-significantly different between AM218W and AM218H, but much higher in AM218M. All experiments were repeated in triplicate with independent samples; error bars represent the SE and asterisks indicate significant differences between different samples (t-test, *P<0.05). (F) Expression analysis of CsWOX1 using mRNA in situ hybridization in developing flower buds of AM218W and AM218M. Letters and numbers in the bottom left corner of images are floral developmental stages according to Fig. 2. (F1–F12) CsWOX1 expression patterns in AM218W male (F1–F6) and female (F7–F12) flowers. In the male flower, CsWOX1 signals were detected in the lateral margin of the petal, the developing anther, the tapetum cell layer, uninuclear pollen, and the arrested carpel. In the female flower, the signals were detected in the lateral margin of the petal, the initial carpel primordium, the developing ovary, ovules, and the cells surrounding ovules. In (F4), the right upper corner is a magnified view of the boxed region in the anther. (F13–F16) In situ hybridization analysis of Cswox1 in male (F13, F14) and female (F15, F16) flower buds of the AM218M mutant. Cswox1 was expressed in the anther and petal at stage 7 (F13), while only in the petal at stage 8 (F14) in the AM218M male flower buds. Cswox1 was expressed in the carpel and petal at stage 7 (F15), while no signal was detected in the carpel at stage 8-3 (F16) in the AM218M female flower buds. Sense probe in the male (F17) and female flower buds (F18) used as negative controls. an lo, anther loculus; an pri, anther primordium; ca, carpel; mi, microsporocytes; nu, nucellus; ov, ovule; pe, petal; pe pri, petal primordium; pl, placenta; st, stamen; ta, tapetum. Bar: 100 µm. (This figure is available in color at JXB online.)
respectively (Supplementary Fig. S3A; Supplementary Table S2). While all 11 CsWOX proteins had the characteristic conserved HD domain (Supplementary Fig. S3B), all seven WUS clade CsWOX proteins (including CsWOX1) shared the conserved WUS box sequence (TLXLFP motif), although that of CsWOX5 was not perfect (Supplementary Fig. S3C). In addition, only CsWUS had an obvious ethylene-responsive element binding factor-associated amphiphilic repression (EAR) domain in the WUS clade.

Expression pattern of CsWOX1/Cswox1 is relevant to floral organ primordia development

CsWOX1 was expressed in the shoots, leaves, and male and female flowers, but not in the roots of the WT, with the highest expression in female flower buds (Fig. 4C). However, CsWOX1/Cswox1 exhibited much higher expression in the mutant than in the WT in all four organs (Fig. 4C). We designed two sets of primers to distinguish the transcript of the mutant than in the WT in all four organs (Fig. 4C). We designed two sets of primers to distinguish the transcript of the mutant than in the WT in all four organs (Fig. 4C). We designed two sets of primers to distinguish the transcript of the mutant than in the WT in all four organs (Fig. 4C). We designed two sets of primers to distinguish the transcript of the mutant than in the WT in all four organs (Fig. 4C). Using the WT-specific primers, transcripts of CsWOX1 were detected in the leaves of two kinds of plants with WT phenotype [homozygous (MfMf) and heterozygous (MfMf)] (Fig. 4D). When using the universal primers, no significant difference in the total mRNA accumulation of CsWOX1/Cswox1 was found between homozygous (MfMf) and heterozygous (MfMf) plants (Fig. 4D, E). These results suggested that CsWOX1 may undergo a negative autoregulatory feedback loop, and a single copy of the WT CsWOX1 transcript could be enough to down-regulate both its own expression and that of the mutant transcript.

mRNA in situ hybridization was performed in male and female flower buds of both the WT and mutant plants. In the WT male flower buds from stage 3 to stage 11, CsWOX1 signals were continually detected in the petal, anther primordium, anther loculus, tapetum, and microsporocytes (Fig. 4F1–F6). In the WT female flower buds, CsWOX1 showed strong expression in the proximal part of the developing carpel primordium at stage 6 (Fig. 4F7), in the placenta at stage 8–1 and 8–2 (Fig. 4F8, F9), and in the ovule primordium at stage 8–3 (Fig. 4F10). At stage 8–4 and stage 9, CsWOX1 mRNA was only detected in the nucellus and the cells surrounding the ovule (Fig. 4F11, F12). In male and female flower buds of the mf mutant, Cswox1 signals were detected in the young petals, similar to the WT, but exhibited a different expression pattern in the stamen and ovary (Fig. 4F13–F16). In the male flower buds, Cswox1 signals were found in the anther primordia before stage 7 (Fig. 4F13), but were undetectable in the abnormal stamens at stage 8 (Fig. 4F14). The Cswox1 signal in the ovary was found in the developing carpel primordium at stage 7 (Fig. 4F15), and disappeared in the abnormally enlarged cavity space at stage 8–3 (Fig. 4F16). The spatial and temporal expression patterns of Cswox1 in the mutant were closely associated with the defective developments of the anther and ovule primordia in the mf mutant (Fig. 2).

The subcellular localization of CsWOX1 and Cswox1 were analysed in onion epidermal cells and Arabidopsis protoplasts. Both proteins were exclusively targeted to the nucleus (Supplementary Fig. S4), which was consistent with the role of CsWOX1 as a transcription regulator, and suggested that the mutant protein was also localized correctly.

Overexpression of CsWOX1 and Cswox1 alleles in Arabidopsis results in abnormal floral morphology and reduced male fertility

Transgenic Arabidopsis plants overexpressing (OEx) CsWOX1 and Cswox1 alleles were developed, and the results are shown in Fig. 5A–D. High levels of ectopic expression of both CsWOX1 and Cswox1 were detected in the transgenic lines (Fig. 5E). Varying degrees of fertility decline were observed among the OEx-CsWOX1 and OEx-Cswox1 independent T3 transgenic lines (Fig. 5A). Of the 21 OEx-CsWOX1 lines, seven had shorter stamens (filaments) and siliques; a similar phenotype was observed in 12 of the 27 OEx-Cswox1 transgenic lines (Fig. 5A–C). The average length of the siliques decreased by 75.4 and 56.9% in the OEx-CsWOX1 and OEx-Cswox1 lines, respectively (Fig. 5F). The pollen grains on these transgenic plants were also abnormal (Fig. 5D). In the most severe cases, more than 73 and 80% of pollens showed degeneration in the OEx-CsWOX1 and OEx-Cswox1 lines, respectively (Fig. 5G). However, in all transgenic lines, the carpel seemed to function normally with almost normal podding after pollination with WT pollen. In addition, there were no obvious differences in other parts of the transgenic plants compared with the WT. Interestingly, no significant difference was found between OEx-CsWOX1 and OEx-Cswox1 transgenic lines in their observed phenotypes (Fig. 5).

Transcriptome profiling reveals key players and regulatory networks in CsWOX1-dependent sporogenesis and flower development

RNA-Seq was carried out for the mf mutant and WT in male flower buds (Supplementary Table S3). The results identified 367 DEGs between the two sets of male flower transcriptomes (Supplementary Table S4). The high quality of the RNA-Seq data was confirmed by a consistent expression trend analysed in the qPCR using 20 selected DEGs (Supplementary Fig. S5). Among the DEGs, a significant portion encoded transcription factors (47 DEGs), or proteins related to hormone signaling (36 DEGs). More than 60 DEGs that were directly related to flower, anther, and pollen development were identified in the male flower transcriptome. (Supplementary Table S4; Supplementary Figs S6, S7).

In the mf mutant male flower transcriptome, WUSCHEL (WUS)-like (Csag6G505860) and AGAMOUS (AG)-LIKE (Csag6G320410) were down-regulated, both of which have critical functions in regulating floral organs development. In floral meristems, the AG gene is thought to be the direct target of WUS. WUS activates the expression of AG, resulting in the formation of staminate and pistillate primordia in the flower center (Lenhard et al., 2001; Lohmann et al., 2001).

The mf mutant male flower transcriptome was highly enriched with down-regulated genes involved in stamen and pollen...
CsWOX1 plays key roles in cucumber flower growth

Among them, NOZZLE/SPOROCYTELESS (NZZ/SPL) is critical for cell differentiation and division of anther cell walls (Schiefthaler et al., 1999; Yang et al., 1999; Liu et al., 2009; Hao et al., 2017). MALE STERILITY 1 (MS1) (Wilson et al., 2001; Ito et al., 2007; Yang et al., 2007) and GAMYB (Preston et al., 2004; Millar and Gubler 2005; Liu et al., 2010) are important for the tapetum and pollen development. Mutation of MYB103 resulted in male sterility because of disruption of callose degeneration, tapetal development, and exine formation (Zhang et al., 2007). In both rice and Arabidopsis, ABORTED MICROSPORES (AMS) and DYSFUNCTIONAL TAPETUM 1 (DYT1) are important for tapetal and microspore development (Sorensen et al., 2003; Zhang et al., 2006). The cucumber homologs of these genes, including CsSPL, CsDYT1, CsMS1, CsAMS, CsGAMYB, and CsMYB103 were down-regulated in the mf mutant (Table 2; Supplementary Table S4).

The RNA-Seq data showed significant enrichment of genes related to phytohormones, which was especially obvious for genes in the auxin signaling pathways (Supplementary Table S4). This observation suggested that auxin signaling might play an important role in CsWOX1-regulated flower growth in cucumber (see below).

We examined the expression patterns of 10 selected DEGs (including CsWOX1, CsWUS, CsAG, and CsSPL) in female flower buds in WT and mf plants (Table 2; Supplementary Fig. S8). All the genes showed consistently down-regulated expression patterns in both male and female flowers of the mf plants. These data suggested that most of these DEGs might play similar and important roles in both male and female flower growth in cucumber.

CsWOX1 physically interacts with CsSPL and Cswox1 affects IAA distribution

In Arabidopsis, NZZ/SPL plays critical roles in sporo-
Balasubramanian and Schneitz, 2000; Causier et al., 2012). The results of Y2H and BiFC assays showed that CsWOX1 physically interacts with CsSPL (Fig. 6A, B), at least in vitro, suggesting that CsWOX1 may regulate sporogenesis in cucumber via the CsSPL-mediated pathway. Surprisingly, the mutant Cswox1 also interacted with CsSPL in yeast cells (Fig. 6A), indicating that the WUS box, which was deleted in Cswox1, may not be necessary for the physical interaction between CsWOX1 and CsSPL. In Arabidopsis, NZZ/SPL functions as an adaptor-like transcriptional repressor in ovule development by recruiting the transcription co-repressor TPL/TPR to inhibit transcriptional repressor activity by acquisition of the WUS box, involving a negative feedback expression regulation (Kieffer et al., 2006; Lin et al., 2013; Zhang et al., 2014; Pi et al., 2015). This accords with data suggested that CsWOX1 might modulate auxin signaling to regulate male and female fertility in cucumber.

**Discussion**

*Mango fruit is a homolog of Arabidopsis WOX1 that plays critical roles in outgrowth of both male and female flowers in cucumber.*

In this study, we identified a spontaneous ‘*mango fruit* (mf)’ mutant in cucumber that exhibited multiple defects in the outgrowth of leaves and flowers, as well as ovule and pollen development. We showed that the *mf* phenotype was caused by a mutation in a homolog of Arabidopsis *WOX1* gene. The roles of *WOX1* genes in the development of lateral organs have been documented from the identification of mutant phenotypes in several plant species (see Introduction). Like other *WOX1* genes, the deduced protein of CsWOX1 had an HD domain and a WUS box motif (Figs 3E, 4A). The WUS box has been shown to be essential for stem cell function for most WUS clade members (Ikeda et al., 2009; Dolzblasz et al., 2016). In fact, the modern clade WOX genes may have evolved for repressor activity by acquisition of the WUS box, involving a negative feedback expression regulation (Kieffer et al., 2006; Lin et al., 2013; Zhang et al., 2014; Pi et al., 2015). This accords with

### Table 2. Selected differentially expressed genes in the mf mutant as compared with the wild-type.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Putative functions</th>
<th>Log2FC</th>
<th>FDR</th>
<th>Arabidopsis ortholog</th>
<th>qPCR validated</th>
</tr>
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<tr>
<td>Csa1G042780</td>
<td>WUSCHEL-related homeobox 1-like</td>
<td>1.54</td>
<td>4.44 × 10^{-6}</td>
<td>WOX1</td>
<td>yes (ff)</td>
</tr>
<tr>
<td>Csa4G436980</td>
<td>Two-component response regulator ARR9-like</td>
<td>1.03</td>
<td>9.74 × 10^{-4}</td>
<td>ARR9</td>
<td>yes (ff)</td>
</tr>
<tr>
<td>Csa1G006300</td>
<td>Two-component response regulator ARR5-like</td>
<td>1.18</td>
<td>1.62 × 10^{-3}</td>
<td>ARR5</td>
<td>yes (ff)</td>
</tr>
<tr>
<td>Csa6G031440</td>
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<td>5.32 × 10^{-4}</td>
<td>ARR18</td>
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</tr>
<tr>
<td>Csa7G434970</td>
<td>Gibberellin 3-beta-dioxxygenase 1-like</td>
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<td>4.14 × 10^{-4}</td>
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</tr>
<tr>
<td>Csa5G172270</td>
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<td></td>
<td>yes (ff)</td>
</tr>
<tr>
<td>Csa6G505860</td>
<td>WUSCHEL-like protein</td>
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<td>2.01 × 10^{-3}</td>
<td>WUS</td>
<td>yes (ff)</td>
</tr>
<tr>
<td>Csa6G520410</td>
<td>Agamous-like MADS-box protein</td>
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<td>6.08 × 10^{-3}</td>
<td>AG</td>
<td>yes (ff)</td>
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<td>Csa3G850670</td>
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<td>5.20 × 10^{-3}</td>
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<td>yes (ff)</td>
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<td>Csa5G601530</td>
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<tr>
<td>Csa5G514470</td>
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<tr>
<td>Csa7G043580</td>
<td>R2R3-MYB transcription factor, putative</td>
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<td>GAMYB</td>
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<tr>
<td>Csa1G008430</td>
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<td>MYB103</td>
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<td>Csa5G391680</td>
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<td>Csa2G200420</td>
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<td>Indole-3-acetic acid-amino synthetase GH3.1-like</td>
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<tr>
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<td>Csa3G731880</td>
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<tr>
<td>Csa3G826860</td>
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</tr>
<tr>
<td>Csa3G852610</td>
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<td>Csa4G001850</td>
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<td>1.00 × 10^{-3}</td>
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<td>Csa4G312880</td>
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<tr>
<td>CsCLV1</td>
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<td>n.a.</td>
<td>CLAVATA</td>
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<td>UR</td>
<td>n.a.</td>
<td>CLAVATA</td>
<td>yes (ff)</td>
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</tbody>
</table>

* a From RNA-Seq.

* b Results are in Supplementary Figs S4, S7.

ff, tested in the female flowers of WT and *mf* mutant; n.a., not applicable; UR, up-regulated in the female flowers of *mf* mutant compared with WT from qPCR analysis.
CsWOX1 plays key roles in cucumber flower growth

the finding in the mf cucumber mutant, in which the truncated protein encoded by the Cswox1 gene lost its WUS box motif (Fig. 4A, B), and the expression of the mutant gene was significantly up-regulated (Fig. 4C–E). Therefore, the expression patterns of CsWOX1 and Cswox1 should be studied.

In situ hybridization revealed similar expression patterns in the WT and mutant cucumber plants in early developing floral organs such as sepals, petals, stamen, and carpel primordia (Fig. 4F). The signals of the mutant gene then disappeared in tissues of defective floral organs (anther and ovule primordia). Subcellular localization assays confirmed the potential transcription factor identity of the WT and mutant protein (Supplementary Fig. S4). In addition, overexpression of the two allele genes driven by the 35S promoter resulted in a similar phenotype in Arabidopsis plants (Fig. 5), suggesting that the truncated protein partially (or fully) functions like the WT protein when expressed at a similar high level. Furthermore, protein–protein interaction assays indicated both the WT and the mutant protein interacted with CsSPL, which was considered as the direct target of CsWOX1 in this study (Fig. 6A, B). These finding suggested that compared with the potential loss-of-function in the mutant protein, elevated expression of the mutant Cswox1 played a critical role in developmental regulation in cucumber and transgenic Arabidopsis.

In Arabidopsis, the gain-of-function mutant wox1-D, which is the result of an enhancer insertion, shows elevated expression...
of WOX1, dwarfed growth, small dark green leaves, failed anther dehiscence, and male sterility; however, the carpel seemed to be fertile (Zhang et al., 2011). In the present study, overexpression of CsWOX1 in Arabidopsis caused no obvious differences in leaf color, plant height, or anther appearance. The moderate decline in male fertility seemed to be the reason for the shortened filaments and/or pollen degeneration. In the cucumber mf mutant, both male and female were sterile, which is the result of defective anther (with normal filaments) and carpel (without ovules) primordia development, respectively (Figs 1, 2). In the petunia maw mutant, female fertility is strongly reduced (Vandenbussche et al., 2009). The different stf mutants of Medicago are all female sterile (Tadege et al., 2011). The garden pea lath mutants often have abnormal carpel development, but no obvious developmental defects in the stamens (Zhuang et al., 2012). However, a wox1 mutant that has both stamen and carpel development simultaneously affected on the same plant is a rare phenotype. The observations of the coordinated expression pattern of the WT CsWOX1 during stamen/carpel development and mutant Cswox1 with the observed phenotypic abnormalities in flowers strongly suggested CsWOX1 functions in the formation of anther and ovule primordia in cucumber.

CsWOX1 may regulate sporogenesis through the CsSPL pathway in cucumber

In higher plants, sporogenesis occurs once the anther/ovule primordia have been established. In Arabidopsis, NZZ/SPL is a key regulator in promoting the formation of megasporocytes and integuments during ovule development, the differentiation of microsporocytes, and stamen identity (Yang et al., 1999; Schiefthaler et al., 1999; Balasubramanian and Schneitz, 2000; Hao et al., 2017). Major players in the NZZ/SPL sporogenesis pathway include various transcription regulators such as AG, TPL/TPR, AMS, MS1, DYT1, TCPs, and the MYB-type transcription factors such as GAMYB and MYB103 (Wilson et al., 2001; Ito et al., 2007). In ovule development, NZZ/SPL functions as an adaptor–like transcriptional repressor, which recruits TPL/TPR co-repressors to inhibit TCP transcription factors for sporocyte differentiation (Chen et al., 2014; Wei et al., 2015). In the stamen, the NZZ/SPL–DYT1–AMS–MS1 pathway is essential for normal tapetal development and pollen production (Wilson et al., 2001; Lou et al., 2018). In the present study, many of the Arabidopsis homolog genes, including CsAG, CsWUS, CsTCP4, CsSPL, CsDYT1, CsAMS, CsGAMYB, CsMYB103, and CsMS1, were down-regulated in the mf mutant, which may be caused by the defective anther primordium (Table 2). This strongly suggested that there is a common gene pathway in cucumber stamen development as well as in Arabidopsis. In addition, Y2H and BiFC assays revealed physical interactions of CsWOX1–CsSPL, Cswox1–CsSPL, CsSPL–CsTCP, and CsSPL–TPL (Fig. 6A, B). Taken together with the observation of similar expression pattern of CsWOX1 and CsSPL in cucumber reproductive tissue (Liu et al., 2018), these results suggested CsSPL as the possible link between CsWOX1’s function in early reproductive organ development and sporogenesis. Thus, based on our knowledge in Arabidopsis and data from the present study, we proposed a working model (Fig. 7, left half) to explain the possible mechanisms by which CsWOX1 controls male and female flower development in cucumber. In this model, CsWOX1 may interact with CsSPL,
which recruits the CsTPL co-repressor to regulate the expression of CsTCPs. The CsWOX1–CsSPL interaction regulates the expression of genes such as CsDYT1, CsAMS, and CsMS1 to control target genes for development of reproductive organs. In a separate study, we found that CsWUS also physically interacts with CsSPL (Liu et al., 2018). Thus, we speculated that there might be competing interactions between CsWOX1 and CsWUS to combine with CsSPL in regulating sporogenesis.

CsWOX1 may regulate outgrowth of floral organs through modulating auxin signaling

Auxin plays an essential role in the determination of the morphology of lateral organs, especially that of leaves and flowers (Leyser, 2002; Cheng et al., 2007). In the mf mutant, many IAA-related or IAA-responsive genes were down-regulated in male and female flowers (Table 2; Supplementary Fig. S8). The IAA distribution inside the male and female flower tissues was also abnormal (Fig. 6C), suggesting that in floral organ outgrowth, CsWOX1 may function via auxin. In Arabidopsis, TPL mediates auxin-dependent transcriptional repression during embryogenesis through physical interaction with indole-3-acetic acid inducible 12/BODENLOS (IAA12/BDL) (Szemenyi et al., 2008). In this study, we showed direct interactions between CsWOX1 and CsSPL, and between CsSPL and CsTPL, which may provide a link between CsWOX1 functions and auxin signaling (Fig. 6A, B). Thus, CsWOX1 might interact with CsSPL to modulate auxin signaling pathways in which CsTPL is involved, and auxin signaling might regulate the outgrowth of floral organs (Fig. 7, right).

Of course, this is a very primitive model, and the first problem that must be answered in future is whether or not the physical interaction between CsWOX1 and CsSPL actually occurs in cucumber (despite the evidence this happens in yeast and heterologous plant systems).

Supplementary data

Supplementary data are available at JXB online.

Supplementary Table S1. Sequence information of the primers used in this study.

Supplementary Table S2. WOX genes identified in the cucumber genome.

Supplementary Table S3. RNA-Seq statistics of flower samples used in this study.

Supplementary Table S4. Differentially expressed genes in male flowers of the mango fruit mutant compared with its WT from the RNA-Seq data.

Acknowledgements

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