A SOC1-like gene MtSOC1a promotes flowering and primary stem elongation in Medicago

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Abstract

Medicago flowering, like that of Arabidopsis, is promoted by vernalization and long days, but alternative mechanisms are predicted because Medicago lacks the key regulators CO and FLC. Three Medicago SOC1-like genes, including MtSOC1a, were previously implicated in flowering control, but no legume soc1 mutants with altered flowering were reported. Here, reverse transcription–quantitative PCR (RT–qPCR) indicated that the timing and magnitude of MtSOC1a expression was regulated by the flowering promoter FTa1, while in situ hybridization indicated that MtSOC1a expression increased in the shoot apical meristem during the floral transition. A Mtsoc1a mutant showed delayed flowering and short primary stems. Overexpression of MtSOC1a partially rescued the flowering of Mtsoc1a, but caused a dramatic increase in primary stem height, well before the transition to flowering. Internode cell length correlated with stem height, indicating that MtSOC1a promotes cell elongation in the primary stem. However, application of gibberellin (GA3) caused stem elongation in both the wild type and Mtsoc1a, indicating that the mutant was not defective in gibberellin responsiveness. These results indicate that MtSOC1a may function as a floral integrator gene and promotes primary stem elongation. Overall, this study suggests that apart from some conservation with the Arabidopsis flowering network, MtSOC1a has a novel role in regulating aspects of shoot architecture.

Keywords: Cell elongation, flowering, FTa1, gibberellins, internode, Medicago, MtSOC1a, MtSOC1b, MtSOC1c, primary shoot axis elongation.

Introduction

The timing of flowering is a critical agronomic trait in plants because of its major effects on reproductive and vegetative productivity (Julier et al., 2007; Jung and Müller, 2009; Yeoh et al., 2011; Tadege et al., 2015). Plants have evolved sophisticated flowering gene networks that integrate different internal and external signals before making the decision to flower (Putterill et al., 2004; Srikanth and Schmid, 2011). In Arabidopsis thaliana (Arabidopsis), the MADS box transcription factor gene SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) and FLOWERING LOCUST (FT) function as key floral integrator genes that promote the transition to flowering (Lee and Lee, 2010; Andrés and Coupland, 2012; Immink et al., 2012; Tao et al., 2012). In inductive long-day (LD) conditions, the FT florigen moves via the phloem to the shoot apex, where it rapidly activates SOC1 expression (Michaels et al., 2005; Turck et al., 2008; Berry and Dean, 2015; Putterill and Varkonyi-Gasic, 2016). SOC1 interacts with AGAMOUS-LIKE 24 (AGL24) and FRUITFULL (FUL) to activate...
LEAFY (LFY), which then triggers the expression of the floral meristem identity gene APETALA 1 (API) and thus floral commitment and the ensuing development of flowers on the flanks of the shoot apical meristem (Lee and Lee, 2010; Balanzà et al., 2014).

The Arabidopsis soc1 mutant is late flowering, and SOC1 overexpression causes Arabidopsis to flower early (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). In addition, analysis of soc1 full double mutants uncovered an overlapping role of these genes in acceleration of flowering time in LDs, floral commitment, meristem determinacy, and in suppressing perennial characters in Arabidopsis (Melzer et al., 2008). AtSOC1 plays a redundant role with AGL24 and SHORT VEGETATIVE PHASE (SVP) in promoting the proliferation of the floral primordium during the early stages of flower development. It is then repressed, but transcript is detected at low levels later in flower development (Immink et al., 2012). Genome-wide studies indicate that AtSOC1 binds detected at low levels later in flower development (Immink et al., 2012). Genome-wide studies indicate that AtSOC1 binds flowering regulatory motifs, particularly in Arabidopsis. AtSOC1 is in the TM3 clade of MIKC+ MADS proteins which is present in all 27 flowering plant genomes analysed (Gramzow and Theißen, 2015), indicative of its general importance. In rice, the SOC1-like gene OsMADS50 promotes flowering while OsMADS56 represses flowering when overexpressed (Lee et al., 2004; Komiyama et al., 2009; Ryu et al., 2009). The FvSOC1 gene also represses flowering in woodland strawberry (Mouhu et al., 2013). In maize, ZmMADS1 promotes the transition to flowering (Alter et al., 2016) and, in petunia, silencing of its three SOC1-like genes delays flowering (Preston et al., 2014). Several SOC1-like genes promote flowering when overexpressed, including orchid DoSOC1 that accelerates flowering in Arabidopsis and orchid (Ding et al., 2013), cotton GhSOC1 (Zhang et al., 2016) that promotes flowering in Arabidopsis, and tree peony PsSOC1 that accelerates tobacco flowering (Wang et al., 2015).

In legumes, a SOC1-like gene, GmGAL1/GmSOC1, from the tropical short-day (SD) plant soybean accelerates flowering when overexpressed in Arabidopsis (Zhong et al., 2012) and Lotus corniculatus (Na et al., 2013). However, loss-of-function soc1 mutations have not yet been associated with altered flowering time for any legume (Fudge et al., 2018). We are studying flowering time regulation in the temperate–climate reference legume Medicago truncatula (Medicago), using its genomic resources including a complete genome sequence and Tnt1 retroelement insertion mutant populations (Tadége et al., 2009; Young et al., 2011; Putterill et al., 2013). Although, like winter annual Arabidopsis, Medicago flowering is induced by vernalization followed by LD photoperiods (Clarkson and Russell, 1975), novel mechanisms of flowering time control are probably present because unlike Arabidopsis, Medicago lacks both the FLOWERING LOCUS C (FLC) clade of floral repressors and the CONSTANS (CO)-mediated photoperiodic switch function (Putterill et al., 2013; Weller and Ortega, 2015).

To dissect the Medicago flowering network, we isolated Medicago flowering time mutants including the early flowering spring mutants (Jaudal et al., 2013; Putterill et al., 2013; Yeoh et al., 2013) and identified flowering time regulators by reverse genetic approaches (Laurie et al., 2011; Jaudal et al., 2014, 2015, 2016). The Medicago FT-like gene, FTa1, strongly promotes the transition to flowering in Medicago and is a major integrator of the floral inductive signals of vernalization and LDs (Laurie et al., 2011). We also uncovered an important role for the predicted Polycomb complex member MtVERNALISATION2 (MtVRN2) as a flowering repressor, repressing FTa1 before vernalization (Jaudal et al., 2016). Thus, MtVRN2 has a different function from Arabidopsis VRN2 which stably represses FLC after vernalization (Berry and Dean, 2015).

Our previous results and other studies (Fudge et al., 2018) also implicated a SOC1-like gene, MtSOC1a, as a target of FTa1 in flowering in Medicago. Transcripts of MtSOC1a accumulated to higher levels in early flowering transgenic Medicago plants overexpressing FTa1 and in the Medicago spring and Mtvrn2 mutants which precociously expressed FTa1 (Jaudal et al., 2013, 2015, 2016; Yeoh et al., 2013). Interestingly, soybean GmSOC1 gene transcript levels were also elevated in early flowering soybean overexpressing an FT-like gene, GmFT2a (Nan et al., 2014).

Here, we focused on characterizing and functionally analyzing the role of MtSOC1a by profiling its temporal and spatial expression, examining Arabidopsis and Medicago transgenic plants ectopically expressing MtSOC1a and determining the phenotype of a Mtsoc1a Tnt1 mutant. These analyses indicated that apart from its capacity to promote flowering, MtSOC1a also promotes elongation of the primary shoot axis of Medicago, a function that has not been shown with SOC1-like genes before. We also profiled the expression of the other two Medicago MtSOC1 genes, MtSOC1b and MtSOC1c, and found that they were strongly dependent on MtFTa1 for their expression as previously observed (Fudge et al., 2018), but had little effect on wild-type Arabidopsis flowering time when overexpressed.

Materials and methods

Bioinformatics

Reciprocal BLASTP searches were used to identify Medicago TM3 clade MADS box proteins in the JCVI Medicago genome (Mt4.0 http://blast.jcvi.org/Medicago-Blast/index.cgi). Additional SOC1 protein sequences were obtained from Hecht et al. (2005) and Gramzow and Theißen (2015) and from JCVI, TAIR (http://www.arabidopsis.org/), NCBI (http://www.ncbi.nlm.nih.gov/), and PHYTOZOME v10 (http://phytozome.jgi.doe.gov) (Goodstein et al., 2012). In silico analysis of normalized RNA sequencing (RNA Seq) data was from the MedCine Medicago truncatula genome database (http://medicmine.jcvi.org/medicmine/begin.do). Medicago Tnt1 lines were identified by screening the FST database (https://medicago-mutant.noble.org/mutant/blast/blast.php).

Plant growth conditions and materials

Growth in controlled environment conditions under cool white fluorescent lights at ~22 °C in LDs (16 h of light/8 h of dark) or SDs (8 h light/16 h dark) with or without prior vernalization was done as described previously (Laurie et al., 2011; Yeoh et al., 2013; Jaudal et al., 2015).
**Medicago truncatula** (Medicago) wild-type R108_C3 (R108) (Trinh et al., 1998), the T1-Tn1 insertion mutant NF3307, and the T1 insertion line (NF1705) were previously reported (Laurie et al., 2011). Tn1 insertion mutants (this work) for MtSOC1a (NF1705) and MtSOC1b (NF7041) in the R108 background were obtained from the Noble Research Institute (Armdrove, OK, USA). The MtSOC1a (NF1705) homozygous mutant was backcrossed once to wild-type R108 and the segregating F$_2$ population were used for linkage analysis and F$_3$ homozygous mutants in some experiments. The 35S::MtSOC1a transgenic plants in the Mtsoc1a mutant background (NF1705) and MtSOC1a mutant (NF1705) regeneration controls (RCs) were generated in this work. The 35S::MtSOC1a (5-1) transgenic line in the Mtsoc1a mutant (NF1705) background was also crossed with wild-type R108, and the F$_1$ and segregating populations were analysed. Genotyping was done using gene-specific and Tn1 primers (see Supplementary Table S1 at JXB online).

Agrobacterium EHA105 with the 35S::MtSOC1a construct (see below) was used to transform leaf tissues of Mtsoc1a. T$_1$ transformants were selected using phosphinothricin and plants were regenerated via somatic embryogenesis as described previously (Corgon et al., 2006; Laurie et al., 2011). Transformed plants were rescued to soil and escapes were eliminated by spraying with Basta herbicide, and genotyping. Flowering time was measured in days after planting and the number of nodes on the primary axis at the time the plant flowered. The length of the primary shoot axis (main stem) was measured from the monofoliate leaf node to the uppermost shoot apical bud.

Epidermal leaf peels were obtained by lightly nicking the stem using a double-edged razor blade, then peeling using fine forceps. Peels were mounted in water on slides and photographed using bright field microscopy (Leica DMR). Plants were sprayed with 10 μM or 100 μM gibberel- lin (dissolved in 0.05% ethanol and 0.02% Tween) or control spray (0.05% ethanol and 0.02% Tween) twice per week unless otherwise specified.

**Results**

MtSOC1a encodes one of four TM3 clade MADS proteins in Medicago

A previous phylogenetic analysis of TM3 MIKC$^+$ MADS proteins, that did not include Medicago proteins, indicated that an ancient gene duplication gave rise to two main branches: branch 1 included Arabidopsis SOC1, AGL14, and AGL19; and branch 2 had AGL42, 71, and 72, all of which are implicated in flowering regulation and other processes (Dorca-Fornell et al., 2011). Our analysis of the Mt4.0 genome (Tang et al., 2014) identified four members; the three SOC1-like proteins MtSOC1a, (Medtr7g075870), MtSOC1b (Medtr8g033250), and MtSOC1c (Medtr8g033220) (Hecht et al., 2005; Gramzow and TheiBen, 2015; Fudge et al., 2018), and Medtr4g102530, more similar to AGL19, but not its top hit in reciprocal BLAST. MtSOC1a–c all shared ~66% amino acid sequence identity with AtSOC1. MtSOC1a was ~66% identical to MtSOC1b and c, while the latter two proteins, encoded by adjacent genes, were 93% identical (Fig. 1A).

Phylogenetic analysis of the MIK domains of TM3-like proteins from temperate legumes (Medicago, pea, chickpea, and red clover), Arabidopsis, and soybean (Fig. 1B) and with additional TM3 proteins (Dorca-Fornell et al., 2011) (Supplementary Fig. S1A) indicated that all four Medicago TM3 proteins were in branch 1, suggesting that branch 2-type proteins had been lost or had diverged in Medicago. Branch 1 was subdivided into a SOC1-like clade with the three MtSOC1 proteins grouping with AtSOC1 and Medtr4g102530 grouping with other AGL14/19-like proteins (Fig. 1B). Within the SOC1 clade, the temperate legumes each had at least one SOC1a protein and one SOC1b/c protein, as recently observed using an IK domain phylogeny (Fudge et al., 2018). These legume SOC1a and SOC1b/c type paralogues probably originated during the whole-genome duplication event that pre-dated speciation of legumes ~58 million years ago (Bell et al., 2010; Young et al., 2011; Fudge et al., 2018). A more recent gene duplication event during speciation of the temperate legumes probably generated the two adjacent paralogues of the ‘b/c’ type gene that are only found in Medicago and alfa\(\text{f}a\) (Fudge et al., 2018).

Based on our *in silico* analysis of normalized RNA Seq data from different tissues (MedicMine *Medicago truncatula* genome database) (Supplementary Fig. S1B), the Medicago TM3 genes have gene-specific expression patterns, but all share the feature of being predominantly expressed in vegetative tissues, as previously observed for the MtSOC1 genes (Fudge et al., 2018). MtSOC1a and MtSOC1c are expressed at similar levels in the aerial tissues; leaf blades and vegetative buds, while MtSOC1b has a different pattern of expression because it is not detected in leaf blades, and only very weakly in vegetative buds.

RNA extraction and reverse transcriptase–quantitative PCR (RT–qPCR) analysis

RT–qPCR was performed on Arabidopsis and Medicago samples using an oligo(dT) primer and SYBR Green PCR Master Mix on an Applied Biosystems 7900HT Sequence Detection System (Laurie et al., 2011; Yeoh et al., 2013; Jaudal et al., 2015). Each data point is derived from the mean of three biological replicates harvested in parallel, with each replicate consisting of a pool of tissues from at least three independent plants. Leaf and shoot apical samples (hand dissected by eye to remove leaves) were harvested separately. The identity of the PCR amplicons was checked by DNA sequencing. Gene expression was normalized to PROTEIN PHOSPHATASE 2A (PP2A) (Medtr8g06260), 35S::MPIM (Medtr8g06260, 35S::MPIM) were generated by inserting the R108 cDNAs into pB2GW7 (Kurmi et al., 2002). Agrobacterium GV3101 containing the pB2GW7 vectors was applied using floral dipping, and transgenic T$_0$ and T$_1$ plants were selected by Basta spraying and genotyping as described previously (Martinez-Trujillo et al., 2004; Jaudal et al., 2015). Flowering time of Arabidopsis plants was measured as the total number of rosette and cauline leaves at flowering.

In situ hybridization

R108 seeds were vernalized at 4 °C for 7 d and then grown in LDs with day and night temperatures of 22 °C and 18 °C, respectively. The plants flowered between 4 and 6 weeks. Shoot apices at both vegetative and reproductive stages of wild-type R108 plants (4 weeks old) were fixed and *in situ* hybridization was performed as described previously (Zhou et al., 2010). A 285 bp cDNA fragment from the non-conserved region of MtSOC1a was used as a probe for hybridization.

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**RT-qPCR analysis of MtSOC1 expression in Medicago**

Recently, the expression of the MtSOC1 genes was shown to be elevated by the environmental cues that induce flowering and regulated by FFl1 (Fudge et al., 2018). Our analysis by RT-qPCR of leaves and shoot apices in time courses, as
opposed to a combination of total aerial tissues and apices in Fudge et al. (2018), gave similar results overall (Supplementary Figs S2–S4).

In different photoperiods, transcript of all three genes was more abundant in LDs than in SDs, with the greatest differential expression observed for *MtSOC1a*, which was highly elevated in the shoot apex of LD plants (Supplementary Fig. S2A, B). In the LD time course (Supplementary Fig. S2C, D), *MtSOC1a* transcript showed an elevation in the leaf and a more dramatic increase in the shoot apex, at 57 d old, prior to flowering. After flowering,
expression dropped in the apex, but continued to rise in the leaf. There was a striking decrease in transcript levels in flower buds and it was further reduced in open flowers, as expected. MtSOC1b had a very different pattern of expression; its transcript was present at low levels during vegetative development, but it showed an abrupt increase after flowering (Supplementary Fig. S2E, F). There was a strong decline in MtSOC1b expression in flower buds, with even lower expression in open flowers. MtSOC1c transcript was present early in young plants and gradually increased through development in both leaves and apices (Supplementary Fig. S2G, H). At 57 d, a pronounced increase in MtSOC1c expression was observed in the apex, similar to MtSOC1a. It was weakly expressed in buds and was greatly reduced in open flowers, a feature it shares with the other MtSOC1 genes.

In a vernalized long day (VLD) time course carried out after vernalizing germinated seeds (Supplementary Fig. S3), the expression patterns of the three MtSOC1 genes and MtPIM, an AP1 orthologue (Beniloch et al., 2006; Cheng et al., 2018), were similar to those observed in LDs, except that more MtPIM expression was detected in the leaves of VLD- than in those of LD-grown plants (Jaudal et al., 2014). None of the four genes was expressed during cold treatment of germinated seeds. In a time course of vernalized ft1 mutant and wild-type seedlings in LDs (Supplementary Fig. S4), MtPIM expression in the ft1 mutant was barely detected, indicating dependence on FTa1 (Laurie et al., 2011). MtPIM transcript levels eventually increased at flowering in the shoot apices, indicating that other factors apart from FTa1 can regulate MtPIM expression (Supplementary Fig. S4A, B) (Laurie et al., 2011). MtSOC1a showed a much slower rate of increase in the ft1 mutant compared with the wild type, indicating that FTa1 is important for the rapid increase of MtSOC1a prior to flowering in the wild type (Supplementary Fig. S4C, D). There was almost no increase in the expression of MtSOC1b and MtSOC1c in the leaves and apices of ft1 mutants, indicating strong dependence of MtSOC1b and MtSOC1c on functional FTa1, in parallel with previous findings (Supplementary Fig. S4E–H) (Fudge et al., 2018). These results indicate that FTa1 is required for the correct temporal pattern of transcript accumulation of the MtSOC1 genes in the wild type. Consistent with this, in 35S:FTa1 transgenic plants, there was a very large increase in the expression levels of MtPIM and strong elevation of the MtSOC1 genes in the shoot apices (Supplementary Fig. S4J). In leaves, MtSOC1a was elevated, while only a modest increase was observed for MtPIM and MtSOC1b, and not much change for MtSOC1c (Supplementary Fig. S4I).

In summary, all the MtSOC1 genes were expressed in SDs, but at a higher level in LDs, and all were dependent to varying degrees on FTa1. MtSOC1a and MtSOC1c both showed steep elevation in transcript levels prior to flowering in the shoot apex, implicating them as candidate regulators of the transition to flowering in Medicago.

**Overexpression of MtSOC1a accelerates wild-type Arabidopsis flowering time**

To investigate if MtSOC1 or MtPIM genes can accelerate flowering in wild-type Arabidopsis, the Medicago genes were fused to the constitutive Cauliflower mosaic virus 35S promoter and transformed into Arabidopsis wild-type Col (Fig. 2).

The 35S:MiPIMT1 transgenic plants had a range of flowering times, with the earliest flowering transfrontants flowering much more rapidly than the wild type (Fig. 2A). The early flowering 35S:MiPIMT plants were much smaller than the wild type, with tiny petioles, leaves, and flowers (Fig. 2F). Premature termination of the inflorescence was also observed due to formation of terminal flowers.

An extremely early flowering phenotype was also observed in four of 15 35S:MtSOC1a transgenic plants (Fig. 2B). The earliest flowering 35S:MtSOC1a transgenic plants were tiny at the time of flowering at 4–5 leaves (Fig. 2E). However, unlike MtPIM and MtFULb (Jaudal et al., 2015), overexpression of MtSOC1a did not result in the formation of terminal flowers. Analysis of MtSOC1a transgene expression by RT-qPCR indicated that the earliest flowering line (P494–26) had the highest levels of transgene expression while the latest flowering line (P494–23), which flowered similarly to the wild type, showed very low levels of transgene expression (Fig. 2B, E). Apart from early flowering, other phenotypes were also observed in some 35S:MtSOC1a plants including smaller plant size, looser flower structure, smaller flowers with shorter petals and sepals, and smaller and thinner siliques with low numbers of seeds that were dark in colour compared with the wild type (Supplementary Fig. S5).

The 35S:MiSOC1b lines flowered similarly to Col wild-type plants (Fig. 2C), although transgene expression was confirmed in selected lines (Fig. 2E). The 35S:MiSOC1b lines also had a similar plant architecture to the wild type (Fig. 2F).

The 35S:MiSOC1c transgenic plants also flowered at a similar time to the wild type (Fig. 2D), with one exception, P496–14 with earlier flowering and elevated MtSOC1c transcript levels relative to the other transgenic plant tested (Fig. 2E). As observed in some 35S:MtSOC1a lines (Supplementary Fig. S5), the earliest flowering 35S:MtSOC1c plant was much shorter in size and produced smaller flowers with curved petals and shorter sepals and smaller siliques with lower yield and dark seeds compared with the wild type.

Recently, Fudge et al. (2018) showed that all three MtSOC1 genes were able to promote flowering of Atsoc1 mutants, in some cases close to wild-type levels when overexpressed, but none flowered earlier than wild-type plants. Here, of the three MtSOC1 genes, overexpression of MtSOC1a appeared to have the strongest effect by dramatically accelerating flowering of four out of 15 transgenic plants compared with zero out of 10 of the 35S:MiSOC1b lines and one out of 17 of the 35S:MiSOC1c lines. The differences that we obtained with 35S:MtSOC1a compared with Fudge et al. (2018) may be due to the two different genetic backgrounds, the numbers of plants analysed, relative level of expression of the transgenes, or growth conditions. MtSOC1a shares 66% amino acid identity with MtSOC1b and MtSOC1c, while the latter two are 93% identical to each other, which raises the possibility that a coding region difference between MtSOC1a and MtSOC1b/c might contribute to the difference between the genes in this assay.
In situ hybridization with MtSOC1a

To analyse the function of MtSOC1a further, we examined the spatial expression pattern of MtSOC1a by *in situ* hybridization of longitudinal sections of shoot apices from 4-week-old VLD plants that had been vernalized for 1 week. These plants flowered when they were between 4 and 6 weeks old. In
Arabidopsis, SOC1 expression is detected in the shoot apical meristem (SAM) and leaf primordia during the transition to flowering, but not in young floral meristems, yet reappears in the centre of the floral meristem at a later stage of floral development (Borner et al., 2000). MtSOC1a was detectable in the vegetative SAM and leaves (Fig. 3A). However, the MtSOC1a transcript level increased in the SAM, leaves, and primary (I1) and secondary (I2) inflorescence meristems at the initial reproductive stages (Fig. 3B, C), while weak expression was detected during the early stages of floral meristem development (Fig. 3D), during differentiation of floral organs (Fig. 3E), and in young flower buds (Fig. 3F).

In summary, our in situ hybridization result is analogous to our RT–qPCR data indicating that MtSOC1a transcript is expressed in Medicago during vegetative development and increases in level in the SAM during the transition to flowering.

MtSOC1a mutant plants are late flowering and have shorter primary stems

To characterize MtSOC1a genetically, we used a mutant line (NF1705) segregating plants homozygous for a Tnt1 retrotransposon insertion in the first exon of MtSOC1a (Fig. 4A) and with greatly reduced expression of MtSOC1a compared with wild-type R108 plants (Fig. 4B).

We crossed the homozygous mutants with wild-type R108 plants and grew an F2 population in VLDs. Out of 206 F2 plants, approximately a quarter (50) were Mtsoc1a Tnt1 homozygotes and late flowering, approximately a half (111) were heterozygotes and flowered like wild-type R108, and approximately a quarter (45) were wild-type segregants and flowered like wild-type R108 (Fig 4C–E). Thus, there was 100% co-segregation between the homozygous Tnt1 insertion in MtSOC1a and the late flowering phenotype. These results indicated that the Tnt1 insertion in MtSOC1a was closely linked to the late flowering locus (within 1 cM) and that the mutation caused a recessive, late flowering phenotype.

In addition to their later flowering, Mtsoc1a mutant plants had a more prostrate appearance (Fig. 4H), because their primary shoot axis was shorter than that of the wild type in LDs and VLDs (Fig. 4H, I). This was associated with reduced internode elongation in the mutant (Fig 4H). Similar results were observed for a segregating population in LD conditions (Supplementary Fig. S6).

To investigate further the flowering response of the Mtsoc1a mutant, it was grown under different photoperiodic conditions with or without vernalization. The mutant flowered later than wild-type R108 plants under all conditions, in both days to flowering and in number of nodes on the primary shoot axis at the time of flowering (Fig. 4F, G). Thus, in summary, knock down of MtSOC1a in Medicago resulted in a delayed flowering time phenotype in the LD and SD conditions tested.

To analyse the molecular basis of the Mtsoc1a late flowering phenotype, candidate target genes were analysed using RT–qPCR in the leaves and shoot apices in VLDs (Supplementary Fig. S7). Only weak effects on gene expression were observed overall. MtSOC1b expression was slightly reduced in the leaves, while MtSOC1c had decreased expression in the leaves and shoot apices of Mtsoc1a. There was also a slight reduction in the transcript levels of MtFULa-b and MtPIM in the leaves of the

**Fig. 3.** In situ localization of MtSOC1a transcripts through the floral transition in longitudinal sections of wild-type R108 plants grown in LDs, after 1 week of vernalization. (A) Vegetative shoot apical meristem (SAM) with leaf primordium (LP) and leaf (L). The inset shows the same section at a lower magnification. (B and C) SAM at the initial reproductive stage. Increased transcript abundance was detected in SAM, L, LP, and primary (I1) and secondary (I2) inflorescence meristems. (D–F) Weak MtSOC1a expression was detected during early stages of floral meristem development (D), during differentiation of floral organs (E), and in young flower buds (F). No expression was detected using a sense control MtSOC1a probe (inset images in C and F). C, carpel; CP, common primordium for petal and stamen; P, petal; SE, sepal; ST, stamen. The black scale bar represents 50 µm. (This figure is available in colour at JXB online.)
Fig. 4. Mtsoc1a mutants have a recessive late flowering phenotype and reduced elongation of the primary stem. (A) Diagram of the MtSOC1a gene with the Tnt1 insertion (black triangle) in the Mtsoc1a mutant. Exons are the black boxes and introns are thin lines. Arrowheads indicate primers. (B) Relative expression of MtSOC1a in 14-day-old R108 and Mtsoc1a seedlings in VLDs. Gene expression was determined using RT-qPCR with 3F and 3R primers, and the data are the mean ± SE of three biological replicates, normalized to Medicago PP2A. Data are presented relative to the highest value. Tissues were harvested 2 h after dawn. (C) Photograph showing PCR genotyping fragments from segregating VLD F2 plants. Plants were scored as early (E) or late (L) flowering relative to R108. Three genotyping primers were pooled in the PCR. (D and E) Graphs showing the flowering time in vernalized LDs (VLDs) scored as the number of days to flowering (D) or the number of nodes on the primary axis at flowering (E) of the F1 progeny (n=14) from a backcross of Mtsoc1a mutants to wild-type R108 plants compared with Mtsoc1a mutants (self-cross) (n=10) and R108 plants (n=13), and the segregating F2 progeny from this backcross (n=206: Mtsoc1a Tnt1 homozygotes, n=50; heterozygotes, n=111; wild-type segregants, n=45) with R108 wild-type control plants (n=28). The data are shown as the mean ± (t.SE) (0.05). (F and G) Graphs showing the flowering time in different conditions including short days (SDs) of Mtsoc1a mutants and R108 scored in either days (F) or nodes to first flower (G). The Mtsoc1a plants in LDs and VLDs were F3 plants after a backcross to R108. The mean ± (t.SE) (0.05) is presented (n=6–50). (H) Photographs of R108 wild-type plants with seed barrels and a prostrate, late flowering Mtsoc1a F2 homozygote with a very short primary axis taken at 45–47 d under VLDs. Arrowheads indicate the primary axis. Scale bar=1 cm. The inset image shows close-up photographs of primary shoot axes of 66-day-old LD wild-type R108 and Mtsoc1a F2 plants. (I) Graph showing the length of the primary axis of Mtsoc1a F2 homozygous mutants (n=39) after a backcross to R108 as compared with wild-type R108 (n=10). The measurements were taken at 45–50 d in VLDs and the data are shown as the mean ± (t.SE) (0.05). (This figure is available in colour at JXB online.)
mutants. All the other genes analysed including the five FT-like genes, FTa1, FTa2, FTc, FTb1, and FTb2, the candidate floral meristem identity gene, LFY; floral identity genes homologous to SEPALATA (SEP3a and SEP3b); and candidate AP2-like genes, TEMPRANILLO (TEM1) and TOE1-like genes did not change much in either tissue of the Mtsoc1a mutants compared with the wild type.

Finally, we asked if mutations in the MtSOC1b/c genes can cause an altered flowering time or plant architecture phenotype. We could not identify a Mtsoc1c knock out mutant, but identified a Mtsoc1b line NF7041 with a homozygous Tnt1 insertion in the second exon of MtSOC1b and gene expression knock down (Supplementary Fig. S8A, B). However, in contrast to Mtsoc1a mutants, the Mtsoc1b Tnt1 insertion line did not display an altered flowering time phenotype or architecture in LD or VLD conditions (Supplementary Fig. S8C–F). A similar result was obtained with two other MtSOC1b Tnt1 mutant lines (Fudge et al., 2018).

Overexpression of MtSOC1a in Medicago causes precocious elongation of the primary stem

In order to investigate the effect of overexpressing MtSOC1a in Medicago, we transformed Mtsoc1a homozygous mutant leaf discs with the 35S: MtSOC1a construct. Transformant plants were regenerated via somatic embryogenesis and planted into soil in LDs. The six independent 35S: MtSOC1a/ Mtsoc1a T0 lines had a range of gene expression levels, with some plants showing much higher expression of MtSOC1a than the regeneration controls (RCs) (Fig. 5C), but they all flowered at a similar time to Mtsoc1a RC at ~67 d with ~21 nodes on average (Fig 5A, B). Analysis of candidate flowering genes in the leaves and apices of transgenic plants also indicated that there was little change in their expression levels compared with RC plants (Supplementary Fig. S9).

Strikingly, however, four T0 transgenic plants had much longer primary shoot axes than RC and wild-type R108 plants (Fig. 5D, E). Thus, the short stem phenotype of the Mtsoc1a mutant was rescued by expression of 35S: MtSOC1a in these transgenic plants. Plants, such as 35S: MtSOC1a (5-1), with the highest level of MtSOC1a expression had the greatest primary shoot axis extension, indicating that primary stem elongation is sensitive to MtSOC1a levels. In contrast, the secondary branches of the 35S: MtSOC1a transgenic plants only displayed a slight increase in internode elongation compared with RCs (inset, Fig. 5E).

Next, we analysed the T1 progeny of 35S: MtSOC1a (5-1) in LD and VLD conditions. In LDs, the primary shoot was again much longer than in R108 wild-type controls, with little effect on flowering time (Fig. 5F–H). The stem elongation was striking because it was seen within the first month of growth and thus occurred well before the transition to flowering at ~80 d. These results indicate that it is possible to separate Medicago primary shoot elongation from the transition to flowering in LDs by ectopically overexpressing MtSOC1a.

In VLDs, in which R108 wild-type plants show much more rapid flowering and primary stem elongation than in LDs (Jaudal et al., 2013), the 35S: MtSOC1a (5-1) T1 plants had a similar primary shoot axis length to vernalized wild-type plants (Fig 5K). In terms of T1 flowering time, the delay in flowering seen in the Mtsoc1a mutant in VLDs was partially rescued (Fig. 5I, J). We observed that the 35S: MtSOC1a T1 plants had longer primary stems in VLDs than in LDs, indicating that they were responsive to vernalization, but this was a reduced response compared with R108 (Fig. 5H, K).

To investigate the effect of the 35S: MtSOC1a transgene in plants without the homozygous Mtsoc1a mutation, we crossed the 35S: MtSOC1a (5-1) line with R108. The VLD F1 plants with the 35S: MtSOC1a transgene flowered at a similar time to wild-type R108 plants, while their primary shoot lengths were slightly longer than those of vernalized wild-type plants (Fig. 5L–N). The same trend was observed in the LD F2 plants, in which the 35S: MtSOC1a plants without the Mtsoc1a mutation had very long primary shoot axes but with flowering time similar to R108 plants. R108 flowered at an average of 56.4 ± 0.68 (t. SE) d (0.05) and the 35S: MtSOC1a F2 segregants flowered at 55.7 ± 3.2 (t. SE) d (0.05).

In summary, the 35S: MtSOC1a transgene partially complements the late flowering of the Mtsoc1a mutant, but does not cause further acceleration of flowering in the MtSOC1a wild-type background. In contrast, the 35S: MtSOC1a transgene fully complements the short stem phenotype of the mutant and gives strikingly elongated primary shoot axes compared with the wild type, particularly in LD conditions.

Increased length of the primary shoot axis in 35S: MtSOC1a plants correlates with increased cell elongation

35S: MtSOC1a transgenic plants produced similar numbers of nodes to Mtsoc1a and wild-type plants in LDs (Fig. 5G), yet had much longer primary stems (Fig. 5H), suggesting that 35S: MtSOC1a promotes internode elongation. To test if cell size was affected, we analysed cell counts within a set area (quadrant) on epidermal peels of stem internodes (Fig. 6A–D).

In LDs, the tall 35S: MtSOC1a (5-1) plants had longer epidermal cells, thus fewer cells/quadrant, than the short Mtsoc1a mutants which had shorter cells (Fig. 6A, B). In VLDs, which promote primary stem elongation in the wild type (Jaudal et al., 2013), the numbers of cells/quadrant were similar between a tall R108 plant and a 35S: MtSOC1a (5-1) plant of similar height, while a younger, shorter R108 plant had smaller cells, thus more cells/quadrant (Fig. 6B). In contrast, the secondary branches of R108 and Mtsoc1a, which were of similar lengths, had similar epidermal cell sizes and thus similar cell counts/quadrant (Fig. 6C, D).

These results indicate that an increase in primary shoot axis length positively correlates with the increased epidermal cell size and that this is promoted by MtSOC1a.

Wild-type and Mtsoc1a plants show increased shoot elongation in response to gibberellin

One of the classical roles of the hormone gibberellin (GA) is to promote stem elongation when applied to dwarf peas (Hedden and Sponsel, 2015). Thus, we hypothesized that the Mtsoc1a
Fig. 5. Overexpression of MtSOC1a in Medicago results in elongation of the primary stem. (A and B) Flowering time in either days (A) or nodes (B) to first flower of individual regeneration controls (RCs) and T0 35S:MtSOC1a plants in LDs. (C) MtSOC1a transcript accumulation in leaves of RC and T0 35S:MtSOC1a plants (32–39 d old) at ZT4. Gene expression was determined using RT–qPCR with data shown as the mean ±SE of three biological replicates, normalized to PP2A and relative to the highest value. (D) Graph showing the length of the primary axis of RC and T0 35S:MtSOC1a plants (53–56 d in LDs). (E) Photographs of wild-type R108, RC, and T0 35S:MtSOC1a plants in LDs, 55–57 d after transfer of germinated seeds (for R108) or regenerated plantlets to soil. Arrowheads indicate the primary axis. The inset image shows close-up photographs of secondary branches from the primary axis of RC and T0 35S:MtSOC1a plants. (F–G, I–J) Graphs showing the flowering time of R108, Mtsoc1a mutants, and T1 35S:MtSOC1a (T1_35S) in LDs and VLDs scored in either days (F, I) or nodes (G, J) to first flower. The mean ± (t.SE) (0.05) is presented (n=7–18). (H, K) Graphs showing the length of the primary axis of T1 35S:MtSOC1a transplants in LDs (H) and VLDs (K) compared with Mtsoc1a and R108 plants. The measurements were taken at 33 d in LDs and 28 d in VLDs. The mean ± (t.SE) (0.05) is presented (n=7–18). The T1 35S:MtSOC1a plants in (F–K) were progeny of T0 35S:MtSOC1a (5-1). (L–N) Graphs showing the flowering time either in days (L) or nodes (M) to first flower and length of the primary axis measured at 28 d (N) in VLDs of the F1 progeny from a cross of T1 35S:MtSOC1a to wild-type R108 (F1_35S) compared with Mtsoc1a and R108 plants. The mean ± (t.SE) (0.05) is presented (n=4–16). The Mtsoc1a plants in (F–N) were F2 homozygous mutants after a backcross to R108. (This figure is available in colour at JXB online.)
**Fig. 6.** *Mtsoc1a* promotes cell and internode elongation in the Medicago primary shoot axis, but is not required for the response to GA$_3$ spray. (A) Photographs of epidermal peels taken from the primary shoot axis of R108 (internode 6), *Mtsoc1a* mutant, and a 35S:*MtsOC1a* F$_2$ plant (internode 6) (from a cross of T, 35S:*MtsOC1a* to wild-type R108) of 31- to 32-day-old plants in LDs. (B) Graphs showing average cell number ± (t.SE) (0.05) within the boxed area (400 μm×50 μm) as shown in (A) on 9–12 epidermal peels of LD plants (left graph) and 5–8 peels of VLD plants (right graph). (C) Photographs of epidermal peels taken from internode 4 of secondary branches of 50-day-old LD R108 and *Mtsoc1a* plants. (D) Graph showing counts of the average cell number ± (t.SE) (0.05) on 5–6 epidermal peels within the boxed area. The scale bars in (A) and (C) are 200 μm. (E and F) Graphs showing the flowering time (E) scored in either days or nodes to first flower and the length of primary axis (F) at 67 d of LD R108 plants sprayed with 100 μM GA$_3$ or control spray. The mean ± (t.SE) (0.05) is presented (n=8). (G) Photographs of 29-day-old LD plants sprayed with 100 μM GA$_3$ (+GA3) or control spray (–GA) with a scale bar of 5 cm. (H and I) Graphs showing the lengths of primary shoot axes (H) and their node number (I) of 33-day-old LD R108, *Mtsoc1a*, and T1 35S:*MtSOC1a* plants sprayed with 10 μM and 100 μM GA$_3$ or control spray. Data are presented as the mean ± (t.SE) (0.05) (n=3–15). (J) Relative expression of *MtSOC1a* at ZT5 in the leaf, shoot apices of the primary and secondary branches (apex), internode 7 of the primary axis (P internode 7), and internode 4 of the longest secondary axis (S internode 4) of 42-day-old LD R108 plants sprayed with 10 μM and 100 μM GA$_3$ or control spray. (K) Relative expression of *GIBBERELLIN 3 BETA-HYDROXYLASE 1-LIKE* (β-HYD, Medtr2g102570) in 42-day-old LD R108 and *Mtsoc1a* plants. Gene expression in (J–K) was determined using RT-qPCR, and data are the mean ±SE of two biological replicates, normalized to PP2A and presented relative to the highest value. The *Mtsoc1a* plants (C, D, G–K) were F$_2$ homozygous mutants after a backcross to R108. (This figure is available in colour at JXB online.)
mutant with its shorter primary stem might have a defect in the GA response pathway. First, we tested the effect of applying 100 μM GA₃ spray weekly to wild-type R108 plants in LDs. This led to a significant increase in primary shoot length (Fig. 6E, G). All plants had a similar node number at this stage (Fig. 6E), indicating that the GA₃ spray had caused internode elongation, but did not promote flowering, with control plants flowering slightly earlier (Fig. 6E).

To compare the effect of GA₃ application, we sprayed plants with GA₃ (10 μM or 100 μM). The primary shoots were measured at 33 d old (Fig. 6H) when the plants had ~10 nodes on the primary shoot axis (Fig. 6I). Strikingly, all genotypes responded to GA₃ with an overall increase in the primary shoot axis length that ranged from ~3-fold (35S: MtSOC1a), ~4-fold (R108), to ~10-fold (Msoc1a) with the 10 μM GA₃ spray compared with the control spray, and with only a minor additional increase with the 100 μM spray. These results indicate that the Msoc1a short primary shoot phenotype does not appear to result from a defect in GA response in LDs.

The results also suggest that GA-mediated primary stem elongation in Medicago does not require MtSOC1a. Consistent with this, MtSOC1a expression in aerial tissues of wild-type plants, including stem internodes, was not elevated by GA₃ application, rather it appeared to be slightly reduced, which may contribute to the slightly delayed flowering observed (Fig. 6I).

One alternative explanation for the short primary shoot stem of the Msoc1a mutant is that the mutant is defective in some aspect of GA production. Therefore, in an initial investigation, we examined the expression of a Medicago homologue of Mendel's pea Le gene that is involved in pea internode elongation. The pea gene encodes a gibberellin 3 beta-hydroxylase involved in the production of bioactive GAs (Lester et al., 1997). However, we did not see any differences in the transcript level of this gene between the Msoc1a mutant and wild type (Fig. 6K).

**Discussion**

The transition to flowering in Medicago is promoted by vernalization and LD photoperiods, and is accompanied by changes in plant architecture including elongation of the primary stem (Chabaud et al., 2006; Jaudal et al., 2013). Our previous studies of Medicago Tnt1 insertion mutants (Jaudal et al., 2013, 2016; Yeoh et al., 2013) identified MtSOC1a as a potential target of FTa1, a potent floral integrator gene (Laurie et al., 2011). However, ours and others recent work indicate that MtSOC1a is one of three SOC1-like genes in Medicago that all depend on MtFTa1 for the magnitude and timing of their expression and can partially complement the Arabidopsis soc1 mutant, raising the possibility that they have redundant roles in flowering (Fudge et al., 2018). We tried to identify Tnt1 mutations in the three MtSOC1 genes. We and others (Fudge et al., 2018), identified several Msoc1b mutants, but none had a flowering time phenotype, while a Msoc1c mutant was not found. However, here we have identified a Msoc1a mutant with flowering time and growth phenotypes indicating that the MtSOC1 genes do not have fully overlapping roles.

In Medicago, our spatial and temporal analysis of MtSOC1a gene expression in aerial tissues indicated that MtSOC1a transcript was highest in the SAM during the transition to flowering, suggesting a potential role in floral induction. Consistent with this, the timing and magnitude of MtSOC1a expression was also partially regulated by Ftα1 as MtSOC1a rapid accumulation was perturbed in the ftα1 null mutants. However, MtSOC1a transcript eventually reached relatively high levels in the leaves and apices of ftα1 mutants at flowering, indicating that other pathways or factors may turn on MtSOC1a expression in the absence of Ftα1, as might be expected of a floral integrator gene (Fudge et al., 2018). MtSOC1a, in turn, does not appear to be the only target of Ftα1 during floral promotion, because ftα1 mutants flower later than Msoc1a mutants and have lost their responsiveness to vernalization (Laurie et al., 2011), unlike Msoc1a mutants.

The phenotype associated with a loss-of-function mutation in a SOC1a-like gene was not previously reported in legumes. Here we showed that MtSOC1a has the capacity to promote flowering in Medicago as shown in a Tnt1 mutant line, which had knock down expression of MtSOC1a and was late flowering in all conditions tested. However, unlike in Arabidopsis, we did not observe precocious flowering in 35S: MtSOC1a Medicago transgenic plants compared with wild-type plants.

MtSOC1a also has an important role in regulating primary shoot axis architecture. A striking feature of the Msoc1a mutants was their very short primary axis, which is a novel phenotype not observed in Arabidopsis soc1 mutants. Overexpression of MtSOC1a reversed this phenotype. Indeed, it caused a dramatic increase in internode elongation and precocious elongation of the primary shoot axis in the transgenic plants, which was not associated with the transition to flowering and not observed in the wild type in LDs. These results indicate that MtSOC1a promotes internode elongation of the primary shoot axis of Medicago, consistent with its expression in stem internode tissue (Fig. 6I) (Fudge et al., 2018).

Previously, we showed that VLD wild-type R108 plants had a much longer primary shoot axis compared with prostrate LD plants (Jaudal et al., 2013). Our analysis of the architecture of the spring early flowering mutants (Jaudal et al., 2013) also indicated that they had a much longer primary shoot axis compared with the wild type, while the late flowering ftα1 null mutants had a very short primary shoot axis (Laurie et al., 2011) comparable with the Msoc1a mutants. These results suggest that the promotion of flowering by Ftα1 is normally tightly linked with primary shoot stem elongation. However, our analysis of the phenotype of 35S: MtSOC1a transgenic plants provides the first evidence that primary shoot axis elongation and acceleration of flowering can be uncoupled in Medicago.

Further investigation of the 35S: MtSOC1a plant phenotype indicates that the increase in internode elongation and thus primary axis height is correlated with an increase in epidermal cell length. This increase in cell length was also observed in wild-type R108 that have elongated primary axes in response to vernalization. Thus, elongation of the primary shoot axis in Medicago is probably due at least in part to increased cell expansion.
Because of the important role of GA in stem elongation in pea and because AtSOC1 is downstream of GA-mediated floral promotion in Arabidopsis (Lee and Lee, 2010), we investigated if the short stem phenotype of Mtsoc1a mutants is due to a defect in responsiveness to GA. GA3 spray promoted stem internode elongation, but not early flowering in wild-type and Mtsoc1a plants in LDs. Thus, the short primary shoot phenotype of the mutant does not appear to result from a defect in GA response. This result also suggests that despite apparent similarities to the effect of 35S:Mtsoc1a, GA spray-mediated primary stem elongation in Medicago does not require functional Mtsoc1a. Consistent with this, Mtsoc1a expression was not elevated by GA3 application. Thus, Mtsoc1a and GA may be involved in different pathways that regulate primary stem elongation in Medicago.

Ectopic expression of the other two MtSOC1 genes, Mtsoc1b and Mtsoc1c, had little effect on flowering time of wild-type Arabidopsis plants in our study but, like Mtsoc1a, they did partially complement the late flowering of the Aso1 mutant, in some cases near to wild-type levels (Fudge et al., 2018). However, Mtsoc1b mutants had no flowering or obvious architectural phenotypes, suggestive of functional redundancy (Fudge et al., 2018). In addition, the Medicago FUL-like genes displayed quite similar patterns of expression and FTL1 dependence (Jaudal et al., 2015). Thus, gene editing approaches in Medicago (Meng et al., 2017) targeting more than one of these genes at a time may serve to optimize future functional studies.

While many SOCI-like genes regulate flowering, others are known to affect other plant processes such as the duration of dormancy in kiwifruit (Voogd et al., 2015). Overall, our study indicates that MtSOC1a regulates Medicago flowering time and primary shoot axis elongation, thus showing perhaps both conservation and divergence with Arabidopsis SOCI function. Since only weak changes in gene expression of candidate flowering time genes were observed in the Mtsoc1a mutant, genomic and biochemical analyses including hormone content may help to illuminate how MtSOC1a executes these roles.

Supplementary Data

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic tree of TM3 proteins and analysis of normalized Medicago RNA Seq data.

Fig. S2. Expression of MtSOC1 genes in LDs and SDs, and overdevelopment in LDs.

Fig. S3. Expression of MtPIM and MtSOC1 genes in VLDs.

Fig. S4. Expression of MtPIM and MtSOC1 genes in fla land 35S:FtTa1 Medicago plants in LD vernalized seedlings.

Fig. S5. Plant architectures of transgenic Arabidopsis plants with 35S:Mtsoc1a transgenes.

Fig. S6. Flowering time and stem lengths of a LD segregating population from R108×Mtsoc1a.

Fig. S7. Gene expression of candidate flowering genes in the Mtsoc1a mutant.

Fig. S8. Mtsoc1b Tnt1 mutant flowered at the same time as the wild type.

Fig. S9. Expression of candidate flowering time genes in T0 35S:Mtsoc1a plants.

Table S1. List of primers.

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