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Article type : Original Article

Shade delays flowering in *Medicago sativa*

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Key words: ARABIDOPSIS THALIANA HOMEOBOX 2 (*ATHB2*), flowering, *Medicago sativa* (alfalfa), PHYTOCHROME INTERACTING FACTOR 3 (*PIF3*), shade avoidance response, SQUAMOSA PROMOTER BINDING PROTEIN LIKE 3 (*SPL3*)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/tpj.14333

ABSTRACT

Shade intolerant plants respond to the decrease in the red (R) to far-red light (FR) ratio (R:FR) occurring under shade by elongating stems and petioles and re-positioning leaves, in a race to out-compete neighbors for the sunlight resource. In some annual species, these shade-avoidance responses (SAS) are accompanied by the early induction of flowering. Anticipated flowering is viewed as a strategy to set seeds before the resources become severely limiting.

Little is known about the molecular mechanisms of SAS in perennial forage crops like alfalfa (*Medicago sativa*). To study SAS in alfalfa, we exposed *alfalfa* plants to simulated shade by supplementing with FR. Low R:FR produced a classical SAS, such as increased internode and petiole length but, unexpectedly, delayed flowering. To understand the molecular mechanisms involved in uncoupling SAS from early flowering, we used a transcriptomic approach. SAS were likely mediated by increased expression of *msPIF3* and *msHB2* in low R:FR. Constitutive expression of these genes in Arabidopsis led to SAS, including early flowering, strongly suggesting their roles are conserved. Delayed flowering was likely to be mediated by the downregulation of *msSPL3*, which promotes flowering in both Arabidopsis and alfalfa. Shade-delayed flowering in alfalfa may be important to extend the vegetative phase under sub-optimal light conditions and thus assure the accumulation of reserves necessary to resume growth after the next season.

INTRODUCTION

Alfalfa (*Medicago sativa*) is a perennial legume used widely around the world as one of the most important forage crops. This is mainly due to its abundant yield, high forage quality, plasticity and capacity to engage in symbiotic associations to fix nitrogen, which makes it an ideal companion crop for other species (Elliott, 1972; Li & Brummer, 2012)

One of the current agronomic goals is to improve the performance of alfalfa plants at high densities, either as pure stands or as a companion crop with grasses, as this would represent an increase in production due to a major number of shoots per unit of area (H. Lin *et al.*, 1999; Varella, 2002; Varella *et al.*, 2010). Since densely grown plants become increasingly mutually shaded, knowing the degree of plasticity in response to crowding signals is of utter importance for future alfalfa improvement.

Green tissues strongly absorb ultraviolet radiation and visible light, including the red-light (R) region of spectrum (around 620 nm), while transmitting and reflecting more effectively in the far-red-light (FR) region (around 730 nm). As a result, the radiation reflected from or transmitted through neighbor vegetation becomes relatively enriched in FR light and bears a low R to FR ratio (R:FR), which is perceived by phytochrome and provides a warning cue of the presence of plant competitors (Casal, 2013; Ballare & Pierik, 2017). Phytochromes have two interconvertible forms: an inactive Pr form, which upon R light absorption is converted into the active Pfr form (Burgie *et al.*, 2014). The Pfr can be rapidly converted back to the Pr form by FR light, or by thermal reversion, which serves as a thermosensing mechanism (Jung *et al.*, 2016a; Legris *et al.*, 2016). "Shade intolerant plants" (Gommers *et al.*, 2013) exposed to low R:FR exhibit elongated stems and petioles which redirect growth to avoid shade, a group of responses known collectively as the "Shade Avoidance Syndrome" (SAS) (Casal, 2012; Ballare & Pierik, 2017). "Shade tolerant plants" mount an opposing response which aims to optimize photosynthesis and other physiological responses under shade (Valladares & Niinemets, 2008; Gommers *et al.*, 2013; Gommers *et al.*, 2017).

Besides increased petiole length and plant height (Schmitt *et al.*, 2003), the SAS include altered pigment biosynthesis involving reduced photosynthesis and chlorophyll (Chl) content (Lichtenthaler *et al.*, 2007; Moon *et al.*, 2008; Cagnola *et al.*, 2012; Li *et al.*, 2014), lower carotenoid levels (Cagnola *et al.*, 2012; Bou-Torrent *et al.*, 2015) and anthocyanin content, depending on the plant species (Steyn *et al.*, 2002; Cagnola *et al.*, 2012; Ding *et al.*, 2016). SAS is also characterized by an upward bending of cotyledons and leaves (hyponasty) (Whitelam & Johnson, 1982; Vandenbussche *et al.*, 2003; Millenaar *et al.*, 2005), and early flowering (Deitzer *et al.*, 1979; Casal *et al.*, 1985; Halliday *et al.*, 1994). The latter is considered to be initiated in order to ensure reproductive success under resource limited conditions (Casal, 2012; Yuan *et al.*, 2017).

Phytochrome B (phyB) is the main repressor of the SAS under high R:FR (Weller & Reid, 1993; Takano *et al.*, 2005; Suzuki *et al.*, 2011; Karve *et al.*, 2012; Sanchez-Lamas *et al.*, 2015). phyB directly regulates transcription factors involved in plant SAS which have been described in *Arabidopsis thaliana* (Devlin *et al.*, 2003; Roig-Villanova *et al.*, 2006). Among them, members of *PHYTOCRHOME INTERACTING FACTOR* family (PIF) (Leivar & Monte, 2014) are positive promoters of SAS and are degraded upon interaction with phyB Pfr (Lorrain *et al.*, 2008; Stephenson *et al.*, 2009; Hersch *et al.*, 2014). Other factors are known to act downstream phys such as the HD-zip transcription factor *ARABIDOPSIS THALIANA*

HOMEOBOX 2 (*ATHB2*), a positive regulator of SAS (Steindler *et al.*, 1999), *CONSTITUTIVE PHOTOMORPHOGENESIS 1* (*COP1*), a negative regulator (McNellis *et al.*, 1994; Pacín *et al.*, 2013) and *FAR INSENSITIVE 219/JAR1*, which regulates multiple shade genes (Swain *et al.*, 2017).

In legumes, SAS studies mostly focused on annual species like *Pisum sativum* (Weller & Reid, 1993; Weller *et al.*, 1995; Weller *et al.*, 1997; Weller *et al.*, 2001) and *Glycine max* (soybean). In *Pisum*, *phyB* mutants showed increased plant height, reduced leaflet area and early flowering (Weller & Reid, 1993). In soybean, shade caused increased internode length, delayed seedling development, reduced branching, total biomass and seed yield (Green-Tracewicz *et al.*, 2011). Interestingly, photosynthesis efficiency increased in soybeans grown under shade, probably as a mechanism of shade tolerance (Gong *et al.*, 2015).

Alfalfa plants grown in the field under different shade treatments (intercropped with trees or using wooden slats) showed a reduction in total dry weight in response to shade (H. Lin *et al.*, 1999; Varella, 2002). Varella et al. (Varella *et al.*, 2010) observed an increase in height and internode length, with a concomitant reduction in the leaf:stem ratio in shade grown plants, implying a reduction in forage quality. Other experiments performed in pure stands of alfalfa showed that growing plants at high density had a positive effect on total biomass, due to a higher number of shoots per unit area (Volenec *et al.*, 1987; Mattera *et al.*, 2013)..

Although physiological assays have been performed to study the SAS in legumes, whether the molecular mechanisms are conserved with model species is still unclear. Recent experiments have been performed in annual legume species employing transcriptomic approaches (Wang *et al.*, 2009). Horvath et al characterized several putative soybean orthologs of shade responsive genes by RNAseq (Horvath *et al.*, 2015). Among others, they identified orthologs of *PHYTOCHROME INTERACTING FACTOR 3 (PIF3)* and *B-BOX PROTEIN 19 (BBX19)*, alongside several heat shock protein orthologs. Another recent study with an annual subterranean clover (*Trifolium subterraneum L*) showed several putative flowering promoting genes were up-regulated under FR enriched light, such as orthologs of *FLOWERING LOCUS T (FT)* and *CONSTANS* like (*COL)* genes (Pazos Navarro *et al.*, 2017).

In this study, we characterized the alfalfa SAS at physiological and molecular levels. Alfalfa plants grown under low R:FR displayed changes in plant architecture and pigment content typical of SAS responses in shade intolerant plants. These changes were likely due to the shade-induced expression of *msPIF3* and *msHB2*, whose role we show is conserved in Arabidopsis. Surprisingly, shade delayed flowering in alfalfa, and we found that it correlated with the down regulation of *msSPL3*. Our data suggest that perennial alfalfa might use a different strategy to annual species; by delaying flowering under suboptimal light conditions, alfalfa may accumulate enough reserves before reproductive stages, which are also necessary to survive the next winter season.

RESULTS

Simulated shade promotes changes in adult plant architecture and in pigment accumulation

To evaluate the effect of simulated shade on plant architecture during adult stages of alfalfa we placed 3-week-old plants (when the first trifoliated leaf was expanded) under W or W+FR conditions and followed their subsequent development (Fig. 1). Simulated shade promoted SAS, as we observed an increase in height (Fig. 1 a,b), petiole length (Fig. 1c) and internode elongation (Fig. 1d,e). To explore the effects of shade on yield and forage composition we measured the dry weight of leaves and stems for shaded vs unshaded plants. Though no differences were seen in biomass yield, shaded plants showed a decrease in total leaf dry weight (Fig. 1f) and a concomitant reduction in the ratio of leaf to stem biomass (Fig. 1g). This reduction is not due to decreased photosynthetic active radiation (PAR) as both treatments received the same PAR, but to decreased R:FR ratio. These data suggest that phytochrome status is important for forage quality, which would be expected to decrease with the reduction of leaf to stem ratios.

We additionally tested two protocols of simulated shade in alfalfa seedlings under SD or LD. We compared the W+FR treatment against a 15 min pulse at the end of each photoperiod to decrease active phytochrome during the subsequent dark period (EOD-FR) (Fig. S1 a-e). We found both protocols to be equally effective to induce SAS in seedlings regardless of photoperiod.

We also tested if shade affected pigment accumulation in alfalfa (Fig. 2). We observed that alfalfa plants grown under simulated shade presented chlorotic leaves (Fig 2a). This correlated well with a reduction of total Chl levels by approximately 25% in leaves of plants grown under shade (Fig. 2b), with the major reduction observed in Chl*b* rather than Chl*a* levels, producing a high Chl*a*:Chl*b* ratio (Fig. 2c,d). Interestingly, carotenoid levels, which have been reported to decrease in low light conditions (Bou-Torrent *et al.*, 2015), were slightly increased under simulated shade (Fig. 2e) while anthocyanins were reduced considerably (Fig. 2f).

Flowering is delayed by both simulated and natural shade

It has been widely reported that several shade intolerant species flower early when grown either under natural or simulated shade as part of the SAS triggered by a low R:FR. Therefore, we analyzed how shade affected flowering in alfalfa (Fig. 3). Contrary to our expectations, alfalfa plants under shade flowered later (Fig. 3a). Flowering was measured as days to flower (Fig. 3c) and as the number of nodes at the moment the first flower appeared (Fig. 3d). By both parameters, shaded alfalfa plants flowered later (Fig. 3a-d). Also, under these conditions flowering plants displayed other SAS phenotypes such as longer internodes confirming these plants were indeed responding to shade signals (Fig. 3e). To rule out that differences in flowering time resulted from variation in the rate of leaf production, we measured leaf appearance over time and found it to be the same in both treatments (Fig. S2). Additionally, we confirmed that this late flowering phenotype was consistent under a range of R:FR ratios (0.8-0.2, Fig. S3).

The data presented above shows that at constant PAR the R:FR ratio controls flowering in alfalfa. However, in natural settings shade leads to decreased R:FR and also to decreased PAR. To investigate the effect of natural shade in flowering, we compared alfalfa plants grown at contrasting planting densities (Fig. S4). Once again, we found that alfalfa plants grown at higher plant density flowered much later than plants grown at low density (Fig. S4 a,b,c). Also, the magnitude of this effect was even stronger that the one we observed under simulated conditions (Fig. 3), which could be due to decreased PAR produced by mutual plant shading.

Transcriptome changes induced by simulated shade

To investigate the downstream mechanisms of SAS in alfalfa we studied changes in the transcriptome induced by shade. The simulated shade conditions used in our experimental design changed the R:FR without affecting other regions of the spectrum. Therefore, we expected that the responses observed were mainly triggered by phytochromes. We performed an exploratory RNA-seq analysis of shaded *vs* unshaded plants. The sequencing produced around 35 million reads per library with around 27 million reads aligned to the MSGI (O'Rourke *et al.*, 2015) (Fig. S5). We identified a cluster of 186 differentially expressed genes (DEGs) with a $\log_2$FC $\geq$ 1 and $\leq$ -1 for upregulated and downregulated genes respectively and a corrected p-value $\leq$ 0.05 (Table S2). Among the primary upregulated genes (Table 1), we identified putative orthologs of *Arabidopsis* shade responsive genes, such as *PIF3, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATHB2), ARABIDOPSIS THALIANA HOMEOBOX 1 (ATHB1), FAR RED INSENSITIVE 219/ JASMONATE RESPONSE 1,* and *FLOWERING PROMOTING FACTOR (FPF1)* (Kania *et al.*, 1997; Ni *et al.*, 1998; Steindler *et al.*, 1999; Lin & Wang, 2004; Capella *et al.*, 2015; Swain *et al.*, 2017). Additionally, orthologs of gibberellin synthase genes (*GA20OX1, GA20OX2*) were upregulated. In particular, *GA20OX1* whose overexpression has been associated with increased cell division and plant growth (Rieu et al 2008, Voorend et al 2016).

An ortholog of *PHYTOENE SYNTHASE (PSY)* was also upregulated by shade, which could account for the higher content of carotenoids we found in shaded plants (Fig. 4E) (Hirschberg, 2001).

The downregulated group of genes (Table 2) presented several orthologs of the light harvesting complex family (*LHCB*) such as *LHCB1.5, LHCB3, LHCB2* (Jansson *et al.*, 1992); as well as genes involved in photosystem II assembly like *PSBP-1* (Yi *et al.*, 2009). In addition, an ortholog of *Arabidopsis GLUCOSE-6-PHOSPHATE TRANSLOCATOR 2 (GPT2)*, a gene that has been tightly associated to dynamic acclimation of photosynthesis (Athanasiou *et al.*, 2010), was strongly downregulated. Likewise, we observed low expression of genes associated to anthocyanin biosynthesis such as *LEUCOANTHOCYANIDIN DIOXYGENASE* (*LDOX)* (Abrahams *et al.*, 2003).

Identification of alfalfa genes involved in SAS

After our initial RNAseq identification of DEGs under shade, we focused on candidate regulators of the alfalfa SAS. Based on the magnitude of expression changes and roles established in *Arabidopsis*, we studied the alfalfa orthologs of *Arabidopsis ATHB2* and *PIF3*, as candidates promoting the SAS (Schena *et al.*, 1993; Ni *et al.*, 1998). *ATHB2* is one of the most prominent characterized players in the *Arabidopsis* SAS, so its role could be conserved in alfalfa (Carabelli *et al.*, 1993; Roig-Villanova *et al.*, 2006; Iannacone *et al.*, 2008; Wang *et al.*, 2016). In the case of *PIF*3, one of its homologs in soybean, *gmPIF3a*, was found to be upregulated in weed shaded plants (Horvath *et al.*, 2015).

Since there is a significant sequence variation among legume species and *Arabidopsis*, we performed a phylogenetic analysis using the complete amino acid sequence of the identified *PIF3* and *HB2* (Fig. 4), comparing them to other putative legume orthologs in order to evaluate their conservation. As expected, the now renamed *msPIF3* grouped nearby other putative legume *PIF3* homologs. This legume *PIF3* clade is a sister to the clade that includes the Arabidopsis *PIF1*, *PIF3*, *PIF4* and *PIF5*, and likely both clades share a common ancestor (Fig. 4a) (Arya *et al.*, 2018). *msHB2* grouped with putative legume orthologs (though branch was unsupported) with *atHB2* as an outgroup (Fig. 4b). We also confirmed the upregulation of both genes under shade independently, by qPCR analysis (Fig. S6a,b).

In order to test whether the roles of *msPIF3* and *msHB2* were conserved, we transformed *Arabidopsis* plants with constructs of *msPIF3* and *msHB2* under the 35S constitutive promoter (Fig. 5). Overexpression of either *msPIF3* or *msHB2* led to a constitutive SAS response in transgenic *Arabidopsis*, including early flowering (Fig 5a,b,c) and elongated hypocotyls (Fig. 5d). Additionally, *msPIF3* overexpressor lines had decreased Chl and carotenoid content (with a low Chl*a*:Chl*b* ratio) (Fig. 5e-g). Therefore, *msPIF3* or *msHB2* overexpression was sufficient to produce a constitutive SAS phenotype in *Arabidopsis*, similar to the responses we observed in shaded alfalfa plants.

The role of *msSPL3* in shade-delayed flowering

Interestingly, overexpression of PIFs in *Arabidopsis* leads to early flowering (Galvao *et al.*, 2015), and we show above that overexpression of *msPIF3* and *msHB2* in Arabidopsis also produce early flowering. These results imply that *msPIF3* and *msHB2* upregulation are unlikely to delay flowering in alfalfa. Therefore, we searched for putative orthologs of *Arabidopsis* flowering genes that could explain the delayed flowering.

Even though we found putative flowering regulators (both promoters and repressors) in the upregulated gene group, such as *AGAMOUS (AG)* (Bowman *et al.*, 1989), *APETALLA1 (AP1)* (Gustafson-Brown *et al.*, 1994), *TEMPRANILLO (TEM1), DIE NEUTRALIS/EARLY FLOWERING FACTOR 4 (ELF4)* (Liew *et al.*, 2009; Sgamma *et al.*, 2014), we found that the expression levels of *SQUAMOSA PROMOTER BINDING LIKE (SPL3)* ortholog, *msSPL3*, was downregulated by more than 60-fold in shaded alfalfa plants (Table 2). We considered *msSPL3* as a strong candidate to explain the shade induced delay of flowering based on previous evidence. *SPL3* in Arabidopsis has been described as an important promoter of phase transition and flowering (Cardon *et al.*, 1997; Jung *et al.*, 2016b) through the upregulation of *FLOWERING LOCUS T*, a prominent flowering promoter in *Arabidopsis thaliana* (Jung *et al.*, 2016b). More importantly, *SPL3* is a conserved target of microRNA 156 and overexpression of microRNA 156 in alfalfa downregulated *msSPL3* mRNA levels and delayed flowering (Gao *et al.*, 2016). Finally, the microRNA 156 genes and their *SPL* targets are well conserved among plants (Poethig, 2013) Therefore, we investigated *msSPL3* as a strong candidate for the shade-induced delay of flowering in alfalfa. First, we confirmed the downregulation of *msSPL3* in shaded plants by qPCR in an independent set of experiments (Table 2 and Fig. 7). Also, *msSPL3* grouped nearby other legume *SPL3* genes, though it was closer to *atSPL6* rather than to the *atSPL3/4/5* clade (Fig. 6a). Therefore, to test if the role of *msSPL3* was conserved, we cloned *msSPL3* cDNA and expressed it constitutively under the 35S promoter in *Arabidopsis* (Fig. 6b-f). After an initial screening, we observed several T1 lines showing an early flowering phenotype (Fig. 6a,b). The overexpression of *msSPL3* resulted in a hastened phase transition, evidenced by a very short vegetative phase with very few leaves (Fig. 6c,d) and an early bolting time (Fig. 6e,f) confirming *msSPL3* as a potent flowering inductor.

Since *SPL3* promotes *FT* expression in *Arabidopsis* (Jung et al 2016), we turned our attention to *FT* homologs in alfalfa. We found that *FT* genes in alfalfa have been poorly characterized, but five *FT* genes could be identified in closely related legume species like *Pisum sativum*

(Hecht *et al.*, 2011) and *Medicago truncatula*: *FTA1*, *FTA2*, *FTB1*, *FTB2* and *FTC* (Laurie *et al.*, 2011) with *FTA1* and *FTB1* as the most likely candidates to induce flowering due to their expression patterns and conserved role as flowering inductors in both species. However, our RNAseq analysis did not show changes for any *FT*-like genes in shaded conditions. We suspected this was probably because these genes are usually expressed later in LD conditions (Laurie et al 2011). So, we analyzed by qPCR *msFTA1* and *msFTB1* expression again at zt5 but also at zt12 and zt16 in W light and W+FR treated plants (Fig. 7). Expression levels of *msFTA1* were similar under both W light and W+FR conditions at zt5 (Fig. 7), while *msFTB1* was not detected. Later in the day, at zt12 we observed a significant reduction of both *msFTA1* and *msFTB1* expression in plants grown under W+FR (Fig.7). At zt16 the expression of *msFTA1* continued to be low under simulated shade, while the decrease of *msFTB1* expression was not observed (Fig. 7). Low expression of *msSPL3* was observed under simulated shade at the three time points tested (5, 12 and 16 hr from lights ON), but only later, at zt12 and zt16, reduced expression of *msFTA1* under shade correlated with decreased *msSPL3* expression (Fig. 7).

***msSPL3* downregulation under shade is partially independent of microRNA 156 in alfalfa**

The microRNA156 target *SPL* genes for degradation. This phenomenon is widely conserved, including legumes as alfalfa (Aung *et al.*, 2015; Gao *et al.*, 2016) and soybean (Sun *et al.*, 2019). Furthermore, Xie and collaborators recently showed that *microRNA156* genes are downregulated in response to shade, resulting in the upregulation of *SPL* genes and early flowering in Arabidopsis (Xie *et al.*, 2017). We hypothetized that shade regulation of microRNA156 could also be part of the response to shade in alfalfa. Therefore we measured the levels of mature microRNA156 at different time points to test if they correlated with *SPL* genes expression (Fig 8). Interestingly, at zt5 the downregulation of *SPL3* by shade was about 60 fold (Fig 7) and we did not observe differences in the levels of mature microRNA156 (Fig 8a), suggesting that shade can induce the downregulation of *SPL3* mRNA by a mechanism largely independent of microRNA156. Similarly, at zt12 we could detect a modest 1.8 fold increase in the abundance of microRNA156 under shade (Fig 8a), while the downregulation of *SPL3* by shade was still maximal, above 60 fold decrease (Fig 7). If the effects were dependent on microRNA156, we would expect similar effects on other targets of

microRNA156, such as *SPL4* and *SPL2* (Aung *et al.*, 2015; Gao *et al.*, 2016). However, we only detected relatively minor changes of *msSPL2* and *msSPL4* mRNA levels in response to shade (Fig 8c,d). Despite we cannot completely rule out a role for the microRNA156 in response to shade, our data indicate that the mechanism to regulate *SPL3* expression in response to shade is partially independent of microRNA156.

Our results, taken together with previous reports on the role of *msSPL3*, strongly suggest that downregulation of *msSPL3* is important to delay flowering of alfalfa in response to shade and suggests a mechanism has emerged in alfalfa to uncouple SAS from flowering induction. The roles of different genes and proteins seem to be conserved in arabidopsis and alfalfa, but in this later species, a mechanism has evolved to downregulate *SPL3* expression in response to shade, contributing to flowering delay.

DISCUSSION

The SAS is an adaptive group of responses that increase fitness in crowded plant stands by reshaping the plant architecture and modifying physiological processes (Schmitt *et al.*, 2003) . Our study shows that, under simulated shade, alfalfa plants induced SAS, which involve, as in other shade intolerant plants, increased plant height, longer petioles and internodes, reduced leaf biomass (Fig. 1), reduced total Chl, reduced anthocyanins and increased carotenoid levels (Fig. 2). Notably, the induction of SAS was accompanied by a delay in flowering time (Fig. 3, S4).

Our transcriptomic analysis of SAS in alfalfa enabled us to reconcile the phenotypes observed with the expression changes of putative orthologs of *Arabidopsis* SAS genes. Putative orthologs of *Arabidopsis PIF3* and *ATBH2*, i.e. *msPIF3* and *msHB2*, were upregulated by simulated shade. To further test their roles, we expressed these genes constitutively in *Arabidopsis*. Both *msPIF3* and *msHB2* were sufficient to induce a constitutive SAS phenotype in *Arabidopsis*, strongly suggesting that their roles are conserved between *Arabidopsis* and alfalfa. Other genes could also contribute to the SAS in alfalfa like the orthologs of *FPF1* (Table 1). In particular, *FPF1* and its cotton ortholog (*ghFPF1*) have

been shown to induce the SAS when overexpressed in *Arabidopsis* (Kania *et al.*, 1997; Melzer *et al.*, 1999; Wang *et al.*, 2014) therefore its role may be conserved in alfalfa as well. Interestingly, *gmPIF3* has been recently shown to be induced in weed-shaded soybeans (Horvath *et al.*, 2015), suggesting that *PIF3* orthologs might play a conserved role in the SAS across legumes.

ATHB family members have also been associated to SAS in *Arabidopsis* and other plant species (Steindler *et al.*, 1999). In tomato, the overexpression of a dominant negative *ATHB2* gene from *Arabidopsis* led to a reduction of SAS (Iannacone *et al.*, 2008). In *Arabidopsis*, *ATHB2* levels are regulated by PIF4 and PIF5 (Lorrain *et al.*, 2008). Whether *msPIF3* acts upstream *msHB2* upregulating its expression is currently unknown and requires further experimentation.

The pigment analysis revealed that the SAS caused a reduction in total Chl levels (Fig. 2). But, contrary to our expectations, the reduction was mostly attributed to a significant decrease in Chlb rather than Chla, which resulted in higher Chla:Chlb ratios in shaded plants (Fig. 2 c,d). Our RNA-seq analysis also accounts for these results, since several members of the *Lhcb* family were downregulated in shaded plants, consistent with the fact that *Arabidopsis* plants with reduced levels of *Lhcb1* and *Lhcb2* have higher Chla:Chlb ratios (Andersson *et al.*, 2003). This may suggest that the Chla:Chlb ratio changes are inherent to some specific species. Also, it could be inferred that the Chla:Chlb ratio should not be solely regarded as an indicator of shaded leaves, since changes could be the result of specific acclimations to different growth limiting conditions.

Regarding other pigments, we found a higher content of carotenoids in shaded plants (Fig. 2e), which could be due to the higher expression (8-fold) of *msPSY*. This is consistent with reports showing increased carotene content in shaded plants (Czeczuga, 1987) and also with reports showing that overexpression of *PSY* genes leads to higher carotenoid content (Busch *et al.*, 2002).

Among all the alfalfa SAS phenotypes we observed, the delay of flowering became the most striking feature (Fig. 3, Fig. S4). Although this phenotype has been previously assayed in particular soybean lines (Cober & Voldeng, 2001), how shade affects flowering

time in iteroparous perennial species has not been deeply analyzed. According to plant strategy theory (Grime, 1977), annual semelparous plants which are common in highly disturbed habitats respond to resource limitations by favoring early and intense reproduction. Contrarily, perennial plants which grow in less disturbed habitats respond to resource limitations by delaying reproduction. Experimental support for this idea was obtained by evaluating flowering time under water limitation of two congeneric annual and perennial species of nettle. *Urtica uren*, a semelparous annual, responded by accelerating reproduction under water stress, whereas *Urtica dioica*, an iteroparous perennial delayed reproduction (Boot *et al.*, 1986). A similar trend was observed when different annual and perennial grasses were subjected to competition and stress and disturbance gradients (Campbell & Grime, 1992). Competition for light resources could also trigger similar diverging responses in iteroparous perennial vs semelparous annual. This proposition has been tested by growing plants with different life histories (semelparous annuals vs iteroparous perennials) under simulated shade. Strikingly, independently of life histories, shade induced reproductive behavior and plants allocated more resources to reproduction at the expense of total biomass and leaf number. (Fazlioglu *et al.*, 2016). Our results show this may not be the case for alfalfa as shade delayed flowering without decreasing biomass, which is in accordance with predictions of the plant strategy theory for iteroparous perennials growing in relatively undisturbed areas. We went further to investigate the molecular nature of this behavior. Our results strongly suggest that downregulation of ms*SPL3* is an important mechanism to delay flowering in alfalfa grown in shade conditions (Table 2, Fig. S6, Fig. 7). In *Arabidopsis*, shade produces high PIF activity which represses mir156 expression, leading to high levels of *SPL* mRNAs, high *FT* levels and accelerated flowering (Xie *et al.*, 2017). Here we found that despite the same orthologs seem to be involved in alfalfa, the opposite modulation of *msSPL3* in response to shade has diversified, consistent with the life history of alfalfa. Under the life history theory, life histories evolved depending on the probability of the adult surviving to the next reproductive event. We propose that by delaying flowering in the shade and accumulating more reserves, alfalfa increases the chance of surviving the next winter and reaching the following reproductive season

EXPERIMENTAL PROCEDURES

Plant material

Seeds of *Medicago sativa* cv Patricia (Fall dormancy 7) were provided by the Instituto Nacional de Tecnología Agropecuaria (INTA) and used in all assays. Seeds were surface sterilized with ethanol 70% followed by SDS/Sodium hypochlorite (1 min each), rinsed with sterile water, dried in a vertical flow cabinet and further treated with chlorine in vapor phase. Seeds were plated in 0,8% agar half strength Murashige and Skoog media (MS) (Murashige & Skoog, 1962) and stratified for 3 days in darkness at 4ºC before transferred to the different experimental conditions.

Shade avoidance assays

For shade assays with seedlings, seeds were plated in magenta boxes and placed in a Percival chamber (Model I30BLL, Percival Scientific, Perry, IA, U.S.A.). The chamber was physically divided in two, both halves sharing same temperature and the same intensity and quality of white (W) light. W light was provided by cool white fluorescent tubes. In the W light supplemented with FR light (W+FR) treatments, FR light was provided laterally by four FR LED lights (Hyper FAR RED – 730nm, LED buy group); both halves were swapped between experiments to rule out any position effect. Photosynthetically active radiation (PAR) registered in the chamber was approximately 100 μmol/m^2.s. Hypocotyl length and appearance and length of the first monofoliate leaf were measured after 7 days, under both long day (LD, 16:8) or short day (SD 8:16) conditions. For the end of day (EOD) experiments, a 15 min light pulse was provided at the end of the respective photoperiod; FR light (15 μmol/m^2.s) for EOD with FR light (EODFR) and R light for controls. For the W+FR assays the resulting R:FR ratio was 0.4. PAR and R:FR were measured using a SpectroSense2 attached with a SKR-1850SS2 light sensor (Skye Instruments).

To test the effect of shade in the architecture and development of alfalfa, seedlings were transferred to individual 1.5 liters pots with a mixture of 3:1:1 soil, perlite and vermiculite supplemented with Red Hakaphos fertilizer (Compo Agricultura, http://www.compo.es). Plants were assorted in trays with a 10 cm distance amongst them and grown for 2 weeks at 23ºC in an incubator under W light and LD conditions until emergence of the first trifoliate

leaf. Next, plants were randomly allocated in two groups, one of them treated with W+FR light, and the other only received W light as control. FR light was provided vertically by two FR lamps. The total PAR registered at soil level was of 109-124 μmol/m^2.s with a R:FR of 7 for the W light treatment and a R:FR of 0.2 for the W+FR light treatment. Plants were grown for two months from the initial transfer to shade conditions.

The length of the main branch, internode length, petiole length and total node number were determined for each plant. At the end of the experiment, stem, petiole and leaf tissue of each plant were dried at 65ºC for 5 days and then weighted.

Flowering time assays

In order to evaluate the effect of shade on flowering time, seedlings were placed in individual 1.5 l pots and grown in an incubator at 23ºC in LD photoperiod under W light until the first trifoliate leaf appeared. Then half of them were supplemented with FR light and the rest remained growing under W light without FR supplementation. The total PAR was 130 μmol m^2 s with a R:FR of 7.47 for the W light conditions and a 0.4-0.6 ratio for the W+FR light conditions. For specific low R:FR ratio assays, Far red was provided laterally and plants were assorted to fixed ratios of R:FR of 0.2 and 0.8.

Flowering time was determined by the appearance of the first flowering bud in the primary stem of each plant. Days to flowering, number of nodes in the primary stem (Sachs, 1999), internode length and the plant height were registered in each condition at the time of flowering.

High vs low density plant growth experiments

To study the effect of plant density on flowering, the same protocol was followed until the first trifoliate leaf appeared, then plants were assorted at a low density per tray (10 cm distance between plants) or at high density (5cm between plants). Days to flowering, number of nodes in the primary stem and the plant height were registered in each condition at the time of flowering.

RNAseq assay

Alfalfa seedlings were grown at 24 °C in LD with W light for 2 weeks and assorted randomly to the two light treatments (W and W+FR). After plants have developed their 6th node (approximately 1 month old plants under W/W+FR treatment), two trifoliate leaves per sample emerging from this 6th node belonging to two different plants were sampled after complete expansion and fast frozen in liquid nitrogen at zt5. Total RNA was extracted with Trizol Reagent (Invitrogen) following the manufacturer's protocols. To estimate the concentration and quality of samples we used NanoDrop 2000c (Thermo Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 NanoKit, respectively. Library preparation and pair-end sequencing with an Illumina HiSeq 1500 were performed at INDEAR, Argentina. Two replicates for each treatment were sequenced and further analyzed.

RNAseq data analysis

Reads obtained were aligned to the *Medicago sativa* Gene index (MSGI v1.2, JA (O'Rourke *et al.*, 2015) using Tophat (Kim *et al.*, 2013). A complete list of normalized counts per million per contig is provided in Table S3. For differential expression analysis a pipeline adapted from ASPli (Mancini *et al.*, 2016) was employed. Edge R was used for p-value and false discovery rate (FDR) correction. Transcripts with at least 0.5 fold change and a FDR corrected p value of 0.05 or less were selected for further analysis. Transcripts were identified by protein Blast (Blastp. version 2.2.25) to databases (NCBI) of mRNA from *M. sativa*, *M. truncatula, Glycine max* and *Arabidopsis thaliana*. RNAseq raw data files were uploaded to Sequence Read Archive (SRA) of NCBI, under the id SUB5088515.

Determination of pigment content

Primary leaves were sampled from the 7th node of 2-month-old alfalfa plants, grown under either W light or W+FR light. For Chl and carotenoid analysis, folioles were weighted and then extracted in dimethyl formamide (Cicarelli) at 4ºC overnight and pigment quantification was determined by using the equations detailed in Wellburn (R. Wellburn, 1994). Anthocyanins were measured as described in Sims (Sims & Gamon, 2002).

Phylogenetic analysis

Amino acid sequences of *msPIF3*, *msHB2* and *msSPL3* were obtained from the MSGI, and putative orthologs sequences from *Glycine max*, *Medicago truncatula*, *Lotus japonicus*, *Cajanus cajan*, *Cicer aerinethum* and *Arabidopsis thaliana* were obtained from GeneBank databases. Sequences were analyzed using MEGA (Tamura *et al.*, 2011). Alignments were performed with MUSCLE (Edgar, 2004) and phylogenetic trees were created using the maximum likelihood method with a bootstrapping of 1000. For each tree, sequences belonging to legumes were obtained from the legume IP website (http://plantgrn.noble.org/LegumeIP/): *Medicago truncatula* (Medtr7g110810.1, Medtr5g013010.1, Medtr2g014200.1, Medtr1g069155), *Glycine max* (Glyma19g40980.1, Glyma11g03850.1, Glyma17g15380.1, Glyma13g31090.1, Glyma15g08270.1, Glyma.02G282100, Glyma.14G032200, Glyma.10G138800, Glyma.19G222000), *Phaseolus vulgaris* (Phvul.002G230300.2, Phvul.003G223200.1, Phvul.008G196800, Phvul.006G028500, Phvul.001G218800, Phvul.007G206000), *Cajanus Cajan* (C.cajan_10677, C.cajan_01609, C.cajan_36197), *Cicer aerietinum* (cicar.ICC4958.Ca_07181, cicar.ICC4958.Ca_22460, cicar.ICC4958.Ca_20229), *Lotus japonicus* (chr6.CM0114.730.r2.m).

Cloning and ectopic expression of *msPIF3, msHB2* and *msSPL3* in *Arabidopsis*

The *msPIF3, msHB2* and *msSPL3* whole cDNAs were PCR amplified from shade treated cDNA samples using primers bearing BamHI, SalI or XbaI restriction sites and cloned in a binary plasmid with these enzymes (New England Biolabs) under the 35S promoter. Constructs were checked by Sanger sequencing (Macrogen, Korea) and introduced into *Arabidopsis thaliana Col-0* plants by floral dip transformation (Clough SJ 1998). Selection of transformants was performed by plating in MS media supplemented with Ammonium glufosinate (Duchefa).

qPCR measurements

Total RNA was extracted from trifoliate leaves at different time points using TRIzol (Sigma) from trifoliate leaves belonging to 45 days old plants treated under W or W+FR.

A total of 2µg of RNA were employed to generate oligo-dT primed cDNAs by using MMLV reverse transcriptase (Life technologies). For the PCR reaction, primers were designed using the corresponding sequences obtained from MSGI (Table S1). qPCRs amplifications were performed using Paq Hot Start DNA polymerase (Stratagene). *msActin2* was used as reference gene (Wang *et al.*, 2015). All determinations were performed on a Roche 480 lightcycler and fold change calculations were performed following the Livak mehod (Livak & Schmittgen, 2001). For microRNA 156 measurements, stem loop qPCR (Varkonyi-Gasic *et al.*, 2007) was performed based on primers and sequences detailed for alfalfa microRNA156 by Aung et al 2015 and Gao et al 2016 (Table S1).

ACKNOWLEDGEMENTS

This work was supported by the Argentine Agencia Nacional de Promoción Científica y Tecnológica (PID 2013-0038). We also want to thank Dr. Julieta Mateos who aided us with microRNA156 measurements.

AUTHOR CONTRIBUTIONS

CDL, CAAD, MV, JJC, MJY and PDC conceived and designed the experiments.

CDL, MSA, MSL, PGG and CEE, performed the experiments.

CDL, JAI and PDC analyzed the data.

CDL and PDC wrote the paper.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Supporting Fig. 1: Effects of two differential shade treatments on hypocotyl length of alfalfa (*Medicago sativa)* seedlings.

Supporting Fig. 2: Leaf appearance measurements of alfalfa (*Medicago sativa*) grown under White (W) or white + Far-red (W+FR).

Supporting Fig. 3: Flowering time measurements of alfalfa (*Medicago sativa*) plants grown under different R:FR ratio.

Supporting Fig. 4: Phenotype and flowering time measurements of alfalfa (*Medicago sativa*) plants grown at a low density (aprox. 10 cm distance) and at a high density (aprox. 5cm distance).

Supporting Fig. 5: Summary of RNAseq results.

Supporting Fig. 6: Validation of DEGs by qPCR in W vs W+FR treated alfalfa (*Medicago sativa*) plants.

Supporting Table 1: Primers used in this study

Supporting Table 2: Complete list of DEGs in W vs W+FR treated alfalfa (*Medicago sativa*) plants.

Supporting Table 3: List of normalized counts per million per contig under W or W+FR.

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Table 1: List of upregulated genes under simulated shade in alfalfa (*Medicago sativa)* identified in the RNA seq. For all genes listed the most likely orthologs name, the orthologs ID, a brief putative function description and the log2 Fold change is provided. All identified genes have a $log_2FC>1$ and an adjusted p-value of $p\leq0.05$.

Table 2: List of downregulated genes under simulated shade in alfalfa (*Medicago sativa)* identified in the RNA seq analysis. For all genes listed the most likely orthologs name, the orthologs ID, a brief putative function description and the log2 fold change is provided. All identified genes have a log2FC>1 and an adjusted p-value of $p\leq0.05$.

Fig. 1: Changes in plant architecture triggered by SAS in alfalfa *(Medicago sativa).* **(a)** Comparison of an adult plant grown under white (W) light or under white supplemented with far-red (W+FR). **(b)** Stem height and **(c)** petiole length of white light vs shaded alfalfa plants. **(d)** Detail of an internode of a W light grown plant (upper panel) or grown under W+FR (lower panel), scale bars represent 1cm. **(e)** Internode length measurements of W vs W+FR grown plants. **(f)** Total leaf dry weight and **(g)** Leaf /stem ratio of dry weights of plants grown under W or W+FR. Bars represent the means $\pm$ SE of 20 individual grown plants per condition. Results were analyzed by T-student tests and asterisk represents different levels of significance ($p\leq0.05$=*, $p\leq0.01$=**, $p\leq0.001$=***).

Fig. 2: Pigment determinations from leaves of plants grown under W (gray bars) or W+FR(black bars) in alfalfa (*Medicago sativa*). **(a)** Phenotype of an unshaded trifoliate leaf (left) vs a shaded one (right). **(b)** Total Chl determination. **(c)** Total Chla and Chlb values and **(d)** ratio of ChA:Chb. **(e)** Total carotenoids and (f) Total anthocyanins. Bars represent the means $\pm$ SE of 15 leaves. Results were analyzed by T-student tests and asterisk represents different levels of significance ($p\leq0.05$=*, $p\leq0.001$=***).

Fig. 3: Flowering time measurements of plants grown under W (gray bars) or W+FR (black bars) in alfalfa *(Medicago sativa).* **(a)** Phenotype of an alfalfa plant grown under W vs W+FR. **(b)** Detailed view of first flowers in W grown plants vs W+FR, white arrows indicate the position of the apical meristem in the shoot. **(c)** Flowering time measured as both days to first flower and **(d)** node to first flower. **(e)** Mean internode length of plants grown under W or W+FR at the time of flowering. Bars represent the means $\pm$ SE of 20 plants per condition. Results were analyzed by T-student tests and asterisk represents different levels of significance. ($p\leq0.001$=***).

Fig. 4: Phylogenetic trees of proteins coded by *msPIF3* **(a)** and *msHB2* **(b)** compared to other legume orthologs of the same putative genes. Different initials stand for: ms (*Medicago sativa*), mt (*Medicago truncatula*), gm (*Glycine max*), pv (*Phaseolus vulgaris*), ca (*Cicer arietinum*), cc (*Cajanus Cajun*), lj (*Lotus japonicus)*, at (*Arabidopsis* thaliana). All trees were developed using the Maximum likelihood method and a bootstrapping of 1000. Bootstrapping values are indicated at each branch.

Fig. 5: Ectopic expression of *msPIF3 and msHB2* in *Arabidopsis thaliana*. **(a)** Phenotype of a wt line transformed with empty vector vs *msPIF3* and *msHB2* overexpressor lines. **(b)** Flowering time measured as total leaf number or **(c)** number of days to bolting, **(d)** mean petiole length, **(e)** total Chl **(f)** Chl*a*:*b* ratio and **(g)** total carotenoids of wt compared to *msPIF3* and *msHB2* lines. Bars represent the means ± SE of 30 T1 plants for the flowering time and petiole length measurements and 15 T1 lines for the pigment measurement assays. Results were analyzed by a one way ANOVA with posterior Dunnett´s test. Asterisks represent different levels of significance. ($p \leq 0.05$=*, $p \leq 0.01$=**, $p \leq 0.001$=***).

Fig. 6: Ectopic expression of *msSPL3* in *Arabidopsis thaliana*. **(a) :** Phylogenetic tree of proteins coded by *msSPL3* compared to other legume orthologs of the same putative genes. **(b)** Phenotype of a T1 selection of wt (right) vs *msSPL3* overexpressor plants (left). **(c)** Detail of an individual wt line vs a *msSPL3* overexpressor line. **(d)** Comparison of leaves of wt vs *msSPL3* overexpressor lines. **(e)** Flowering time measured as total leaf number or **(f)** number of days to bolting. Bars represent the means ± SE of 30 T1 plants per condition. Results were analyzed by T-student tests and asterisk number represents different levels of significance. ($p \leq 0.001$=***). Different initials stand for: ms (*Medicago sativa*), mt (*Medicago truncatula*), gm (*Glycine max*), pv (*Phaseolus vulgaris*), ca (*Cicer arietinum*), cc (*Cajanus Cajun*), lj (*Lotus japonicas)*, at (*Arabidopsis thaliana*). All trees were developed using the Maximum likelihood method and a bootstrapping of 1000. Bootstrapping values are indicated at each branch.

Fig. 7: *msSPL3*, *msFTA1* and *msFTB1* mRNA levels of adult plants grown under W vs W+FR. Trifoliate leaves belonging to adult alfalfa plants grown under either W or W+FR were harvested at zt5 (left panel), zt12 (middle planel) and zt16 (right panel), and the indicated genes (abscissas) measured by qPCR. Bars represent the means ± SE of 3 biological replicates consisting of 1 trifoliate leaf per 45 days old plant treated under W or W+FR. Results were analyzed by a one way ANOVA with posterior Dunnett´s test. Asterisks represent different levels of significance. ($p \leq 0.05$=*, $p \leq 0.01$=**).

Fig. 8: Expression levels of microRNA 156 and its targets *msSPL2-msSPL4* in plants grown under W vs W+FR. Trifoliate leaves belonging to adult alfalfa plants grown under either W or W+FR were harvested at zt5 (left panels) or zt12 (right panels), and *microRNA156* (a), *msSPL2* (b) and *msSPL4* (c) were measured by qPCR. Bars represent the means ± SE of 4-6 biological replicates, each consisting of 1 trifoliate leaf per 45 days old plant treated under W or W+FR. Results were analyzed by a one way ANOVA with posterior Dunnett´s test. Asterisks represent different levels of significance. ($p \leq 0.05$=*).

Gene	Orthologous ID	Putative Roles	Log FC
PIF3	Medtr7g110810.1, AT1G09530.2	Shade promoter	5.9847
ATHB-2	Medtr5g013010.1,AT4G16780.1	Shade promoter	2.6787
ATHB-1	Medtr5g038280.1,AT3G01470.1	Shade promoter	1.3135
FPF1	Medtr1g009900.1,AT5G10625.1	Shade promoter/ flowering promoter	4.3042
TEM1	Medtr1g093600.1,AT1G25560.1	Flowering Repressor	1.3233
DNE/ ELF4	Medtr3g070490.1,AT2G40080.1	Flowering Repressor	1.7608
mtCOLd	Medtr4g128930.1,AT5G57660.1	Non determined	1.1126
ILA	Medtr7g116425.1,AT1G64790.1	Required for systemic acquired resistance	2.3169
JAR1/FIN219	Medtr7g117110.1,AT2G46370.4	Modulation of Shade avoidance response	5.2214
IMPA-4	AT1G09270.3	Mediates nuclear protein import	4.5586
PSY	Medtr4g107290.1,AT5G17230.3	Carotenoid biosyntesis	2.7707
ANX2	Medtr4g052290.1,AT5G28680.1	Supression of ABA signalling	3.7128
CKX6	Medtr3g036100.1,AT3G63440.1	Catalyzes the oxidation of citokynines	3.6736
GA20OX1	Medtr6g464620.1,AT4G25420.1	Gibberellin biosyntesis	1.7547
GA20OX2	Medtr3g096500.1,AT5G51810.1	Gibberellin biosyntesis	2.2744
JMT	Medtr1g022465.1,AT1G19640.1	Jasmonate biosyntesis	1.5273
HAB1 LIKE	AT1G72770.3	ABA signalling	1.0761
TPS02	Medtr2g089130.1,AT4G16730.1	Terpene biosyntesis	3.9512

Table 1

Gene	Orthologous ID	Putative Roles	Log FC
TPX2	Medtr6g032995.1,AT3G23090.2	Negative regulator of hypocotyl cell elongation in the light	-3.5671
LHCB3	AT5G54270.1	Photosyntesis promotion	-2.1796
ELIP1	Medtr1g102780.1,AT3G22840.1	Photosyntesis regulation	-1.8300
LHCB1.5	AT2G34420.1	Photosyntesis promotion	-1.7966
Photosystem II 5 kD protein	Medtr3g030850.1,AT1G51400.1	Photosystem II regulation	-1.7947
LHCB2	Medtr6g012080.1,AT2G05100.1	Photosyntesis promotion	-1.4812
SPL3	Medtr2g014200.1,AT2G33810.1	Strong vegetative phase change and flowering promoter	-6.5017
CYP714A1	Medtr0147s0030.1,AT5G24910.1	Inactivation of early GA intermediates.	-6.1178
PRT6	Medtr7g061540.1,AT5G02310.1	Ubiquitin ligase	-12.3889
MC5	Medtr0340s0030.1,AT1G79330.1	Modulation of programmed cell death	-9.9573
GSTF11	Medtr3g064700.1,AT3G03190.1	Conjugation of reduced glutathione	-6.5334
GPT2	Medtr2g022700.1,AT1G61800.1	Required for dynamic acclimation of photosynthesis	-3.7291
PHB3	Medtr5g093030.1,AT5G40770.1	Ethilene response modulation	-13.2134
LDOX	Medtr5g011250.1,AT4G22880.2	Involved in anthocyanin and protoanthocyanidin biosynthesis	-9.1434
HCT	Medtr8g075610.1,AT5G48930.1	Influence on the accumulation of flavonoids which inhibit auxin transport.	-8.3434
ATMES1	Medtr5g018365.1,AT2G23620.1	Conversion of methyl salicylate (MeSA) to salicylic acid (SA)	-3.7007
IPT3	Medtr1g072540.1,AT3G63110.1	Involved in cytokinin biosynthesis	-1.2807
CAP160	Medtr1g100627.1,AT4G25580.1	Cold acclimatation protein	-5.3210

Table 2