Nitrates acts at the *Arabidopsis thaliana* shoot apical meristem to regulate flowering time

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Summary

- Optimal timing of flowering, a major determinant for crop productivity, is controlled by environmental and endogenous cues. Nutrients are known to modify flowering time; however, our understanding of how nutrients interact with the known pathways, especially at the shoot apical meristem (SAM), is still incomplete. Given the negative side-effects of nitrogen fertilization, it is essential to understand its mode of action for sustainable crop production.
- We investigated how a moderate restriction by nitrate is integrated into the flowering network at the SAM, to which plants can adapt without stress symptoms.
- This condition delays flowering by decreasing expression of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) at the SAM. Measurements of nitrate and the responses of nitrate-responsive genes suggest that nitrate functions as a signal at the SAM. The transcription factors NIN-LIKE PROTEIN 7 (NLP7) and NLP6, which act as master regulators of nitrate signaling by binding to nitrate-responsive elements (NREs), are expressed at the SAM and flowering is delayed in single and double mutants. Two upstream regulators of SOC1 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 (SPL3) and SPL5) contain functional NREs in their promoters.
- Our results point at a tissue-specific, nitrate-mediated flowering time control in *Arabidopsis thaliana*.

Introduction

Plants take up nutrients from soil throughout their life cycle, providing the potential to grow in tight coordination with the developmental demand (Krouk *et al.*, 2011). This is crucial for the timely transition between developmental phases such as the switch from the vegetative to the reproductive phase (Lin & Tsay, 2017). Nitrogen (N) is an essential macronutrient for plant growth, and high yields of almost all cultivated crops require application of large amounts of fertilizer. Hence, N plays an important role in agriculture to improve yield and increase agronomical productivity. However, although fertilizers have helped to increase yield, they also have negative effects, causing environmental and human health problems as well as decreased biodiversity (Smil, 1999; Good & Beatty, 2011; Shibata *et al.*, 2015). A surplus of synthetic fertilizers and manure causes emission of nitrous oxide into the atmosphere and leaching of nitrate into soil patches. Many natural water basins and aquifers around the world are currently steering towards severe nitrate pollution through long and continued application of organic and synthetic fertilizers. The maximum contaminant level for public water supply of 50 mg nitrate l⁻¹ determined by the World Health Organization (or 10 mg l⁻¹ nitrate-N, stipulated by the US Environmental Protection Agency) is being well exceeded (Ward *et al.*, 2018). Reducing the application of fertilizers is a general aim of modern agriculture.

Nitrate is the major source of N for plants and, once taken up by the roots, is distributed within plants by a large number of nitrate transporters. Besides being a nutrient, nitrate itself acts as a signal regulating directly the expression of hundreds of genes (reviewed in Noguero & Lacombe, 2016). These genes encode proteins required for nitrate transport and assimilation, and the reprogramming of carbon (C) and N metabolism, as well as transcription factors and regulatory proteins, triggering a cascade of changes that support increased growth. An early event in nitrate responses involves the accumulation of the transcription factor NIN-LIKE PROTEIN 7 (NLP7) and its close homolog, NLP6, in the nucleus (Marchive *et al.*, 2013; Guan *et al.*, 2017), where they bind to nitrate-responsive elements (NREs) in promoter regions of target genes (Konishi & Yanagisawa, 2013) and regulate their expression.

Yield depends not only on vegetative growth, but also on the optimal timing of reproductive growth. The latter starts with the...
transition of the shoot apical meristem (SAM) from a vegetative into an inflorescence meristem. This developmental switch is regulated by a complex hierarchical signaling network that integrates many environmental and endogenous stimuli (Blumel et al., 2015). Within this network, core pathways have been described that orchestrate responses to day length and light quality (photoperiod pathway), fluctuations in temperature (ambient temperature pathway), exposure to longer periods of cold (vernalization), gibberellic acid signaling (GA pathway), endogenous regulators independent of light and GA (autonomous pathway), and the plant’s age (age pathway). Although these signaling pathways have been extensively studied, knowledge of how metabolic signals regulate flowering lags behind. A notable exception is the dependence of the induction of flowering on a plant’s energy status. The trehalose 6-phosphate (T6P) pathway has been shown to convey information about the sucrose status to the flowering network at two signal perception sites. In the leaves, it is necessary and sufficient to induce FLOWERING LOCUS T (FT), also known as the florigen and a member of the photoperiod pathway, at the end of long days. At the SAM, the T6P pathway interacts with the age pathway both via miR156 and independently of it (Wahl et al., 2013).

The nitrate supply is known to modify several developmental processes, including flowering time (Rideout et al., 1992; Corbesier et al., 2002; Castro Marín et al., 2011; Liu et al., 2013). The first evidence suggesting that nitrate might be involved in the regulation of flowering time in Arabidopsis thaliana was obtained from genetic studies showing that nitrate assimilation and signaling/uptake mutants are late-flowering (Tocquin et al., 2003; Seligman et al., 2008). However, interpretation of these observations is challenging as these mutants display severe global metabolic changes, including sugar content, all of which can affect flowering time (Scheible et al., 1997; Klein et al., 2000). Indeed, studies conducted with A. thaliana under various conditions reveal that nitrate has contrasting effects on flowering time depending on the growth system (agar-, vermiculite-, soil-based) and the source of nitrate used (KNO₃, NH₄NO₃, mixed, supplemented with glutamine (Castro Marín et al., 2011; Kant et al., 2011; Liu et al., 2013; Yuan et al., 2016; Lin & Tsay, 2017; Gras et al., 2018)). Hence, depending on the study, conflicting results were obtained regarding which flowering genes show differential expression in different N regimes, or which flowering pathway mutants alter the response to N, whereas various flowering genes have been reported as correlating with the growth system used but not necessarily responding to nitrate directly (Lin & Tsay, 2017). In a review on the current status of research regarding N-dependent control of flowering, Lin & Tsay (2017) compiled data published by early 2017 and proposed a U-shaped response of flowering to nitrate, with an optimal nitrate concentration range for flowering, which is delayed by higher and suboptimal nitrate concentrations (Lin & Tsay, 2017). In addition, flowering is promoted by nitrate starvation (Lin & Tsay, 2017), with the latter being an extreme condition revealing a plant’s escape strategy when exposed to stress. Acute stress can either induce or delay flowering (Kazan & Lyons, 2016; Takeno, 2016), defining an emergency exit to secure next generations, should the environmental conditions be too harsh to allow adaptation. A recent review (Takeno, 2016) suggests a separate pathway for stress-induced flowering, which responds to diverse unsuitable conditions (e.g. poor nutrition, UV light exposure or drought). All these flowering-inducing ‘stresses’ have in common the fact that their corresponding signals, as different as they might be, converge with the flowering network in leaves at the level of FT. Taking this into account, Castro Marín et al. (2011) supplemented their agar growth system with 4 mM glutamine, in order to be able to vary nitrate but avoid acute N starvation.

Optimal nitrate concentrations differ between growth systems and for species. Furthermore, the concentrations are likely to vary between various tissue types. This might explain why various research groups have proposed different perception sites for nitrate in the flowering time network (Castro Marín et al., 2011; Liu et al., 2013; Gras et al., 2018). Additionally, most previous studies were restricted to analyses on whole rosettes or seedlings instead of tissue-targeted approaches (e.g. in the SAM) and reflect a great variability in the light and temperature regimes used to grow the plants. Previous research provided evidence that nitrate, once taken up by plants, might interfere with the known flowering network in leaves to modify the flowering time, as a set of flowering genes expressed in leaves (e.g. FT, SMZ, SNZ) was affected by the N status (Castro Marín et al., 2011; Lin & Tsay, 2017; Gras et al., 2018). However, no evidence has been provided on whether nitrate enters and operates at the SAM.

Tschoep et al. (2009) established an N-limited soil system, which involves growth of plants in an ‘optimal’, full-nutrition (ON) or ‘low’ nitrate (LN) soil. The concentration of the latter was chosen in such a way that plants were able to adapt to the conditions and maintained a reduced but constant growth over several weeks. This was reflected by the metabolic phenotype, with similar amounts of protein to ON-grown plants (hereafter ON plants). Analyses of other nutrients, which might potentially affect growth and flowering time (e.g. phosphate or sulfate), demonstrated that their content was unaltered. LN-grown plants (hereafter LN plants) did not show any visible stress symptoms, such as anthocyanin accumulation, and ON-grown plants flowered at a similar time to wild-type (Col-0) plants on standard soil. Hence, this growth system is an ideal one for studying the effects that N availability might have on flowering time in A. thaliana.

Here, we report on a study that made use of the growth system established by Tschoep et al. (2009). We found that nitrate is present in the SAM and that nitrate-regulated genes involved in N assimilation respond in the SAM to N availability. Our data indicate that nitrate acts on the expression of components of the age pathway (i.e. SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3) and SPL5) via NRE motifs in their promoters to induce flowering, and that this process involves the NLP transcription factors NLP6 and NLP7 in the SAM, and leads to differential expression of the flowering integrator gene SOC1.
Materials and Methods

Plant material

The plants used in this study were *A. thaliana* Columbia (Col-0, CS76778) wild-type and mutant and transgenic lines such as co-10, fdl-3, soc1-6, ft-10, tif-1, ft-10/tif-1, 35S::amiRTTPS1 35S::MIR156, nlp7-1 and nlp7-1 nlp6-2 (Michaels & Amasino, 2001; Rosso et al., 2003; Abe et al., 2005; Michaels et al., 2005; Schwab et al., 2005; Yoo et al., 2005; Laubinger et al., 2006; Lee et al., 2007; Jang et al., 2008, 2009; J. W. Wang et al., 2009; Wahl et al., 2013). Genotypes were confirmed (Supporting Information Table S1). Cassettes containing synthetic promoters with four copies of the respective NRE (gene synthesis; Eurofins, Ebersberg, Germany) were designed as previously described (Konishi & Yanagisawa, 2010).

Growth conditions

Plants were grown in growth chambers (Percival Scientific Inc., Perry, IA, USA) at 22°C under long-day (LD; 16 h : 8 h, light : dark) or short-day (SD; 8 h : 16 h, light : dark) conditions with 160 μmol m⁻² s⁻¹ light intensity (Philips F17T8/TL841/Alto). A shift from SD to LD conditions was used to induce flowering as previously described (Schmid et al., 2003).

A modified soil-based N-limited growth system (ON or LN) was used to grow plants (Tsoeop et al., 2009). Soil mixtures were stored for at least 2 wk at 10°C before use. The ON and LN soils contained c. 31.5 and 1.25 mg inorganic N per 6 cm pot (100 ml or 36 g soil per pot), respectively.

Phenotypic analyses

Flowering time was defined as days to bolting (DTB) and the total number of leaves (TLN) (Table S2). At least 11 genetically identical plants were used to determine flowering time. Student’s *t*-test was used to test the significance of differences. The leaf initiation rate was calculated by dividing TLN by DTB or recording the number of visible leaves (> 2 mm) (Wang et al., 2008). Juvenile leaves were defined as leaves without abaxial trichomes (Telfer et al., 1997).

qRT-PCR

The total RNA was extracted using a modified phenol : chloroform : isooamy1 alcohol (25 : 24 : 1) method as previously described (Wan & Wilkins, 1994). cDNA preparation and quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurements were performed as previously described (Wahl et al., 2013). qRT-PCR analyses were performed on two to four biological replicates with three or four technical replicates using the Power SYBR® Green-PCR Master Mix (Applied Biosystems/Life Technologies, Waltham, MA, USA). Relative quantification of gene expression was performed using a comparative cycle threshold (CT) method (Livak & Schmittgen, 2001) with a reference gene index (RGI) and presented as previously described (Wahl et al., 2013). Primer sequences are listed in Table S1.

For *GUS* reporter gene detection, seedlings were harvested in 90% ice-cold acetone, and washed with and incubated in staining buffer (10% Triton X-100, 0.5 M NaPO₄, 100 mM K-ferrocyanide, 100 mM ferricyanide, 100 mM X-Gluc) overnight at 37°C. Seedlings were dehydrated in an ethanol series, fixed with FAA (formaldehyde, ethanol, acetic acid), washed in 70% ethanol before being embedded in wax. For RNA *in situ* hybridization, apices of plants were collected at the end of the day, fixed and embedded using a Leica system (ASP3005, EG1160; Wetzlar, Germany). Sections of 8 μm thickness were prepared using a rotary microtome (RM2265; Leica). Probes were generated from cDNAs as previously described (Wahl et al., 2013). For LFY, SOC1, SPL3, SPL4 and SPL5, full open reading frames containing plasmids were provided by Weigel’s and Schmid’s laboratories (Weigel et al., 1992; R. Wang et al., 2009). RNA *in situ* hybridizations and toluidine blue staining was carried out as previously described (Wahl et al., 2013).

Search for NREs in core flowering time genes

Nitrate-responsive elements (tGACcCTTNₙAAGagtcc) (Konishi & Yanagisawa, 2010) were searched in the sequences of upstream intergenic regions of known flowering genes with a maximum distance of 3000 bp to the ATG codon. A list of putative NREs is presented in Table S3 and all genes included in the analysis are provided in Table S4.

Metabolite and nitrate reductase activity measurement

For metabolite measurements, plants were harvested at end of the day and metabolites were analyzed as previously described (Scheible et al., 1997; Nunes-Nesi et al., 2007). Nitrate was measured in a coupled enzymatic assay (Cross et al., 2006). An adapted protocol was used for SAM samples, from which nitrate was extracted with 70% ethanol once (Stitt et al., 1989). Total protein was extracted as previously described (Hendriks et al., 2003). Enzyme extraction was performed with some modifications of the buffer ingredients (without triton and BSA) (Gibon et al., 2004). The extract was also used for the measurement of total proteins, which were assayed with the Bio-Rad Bradford reagent according to the manufacturer’s instruction (Bio-Rad). For T6P measurement, tissue samples were extracted and measured as previously described (Lunn et al., 2006).

Results

Flowering is delayed when nitrate supply is suboptimal

We used a N-limited soil system (Tsoeop et al., 2009) (Fig. 1a). ON contains nitrate at a comparable concentration to standard full-nutrition soil (31.5 mg N per pot) and supports the normal life cycle of wild-type plants (Tsoeop et al., 2009). Flowering times for ON-grown wild-type plants (Table S2) are comparable.
with data from studies using standard full-nutrition soil (± 21 d after germination (DAG) in LD and 61 DAG in SD conditions (Hartmann et al., 2000; Lim et al., 2004; Kim et al., 2007; Wahl et al., 2013), demonstrating that the selected nitrate concentration in ON is optimal for plant growth.

Plants growing in LN (1.25 mg N per pot) did not display any stress symptoms (e.g. chlorotic leaves or decreased protein and amino acid contents (Figs 1b,c, S1a)), which usually mask direct N-dependent responses (Tschoep et al., 2009). Expression analyses of a stress-responsive gene by qRT-PCR did not result in any significant changes between ON and LN plants (Rowan et al., 2009) (Fig. S1b). Compared with ON plants, rosettes of LN plants have significantly lower nitrate concentrations (Fig. 1d), are smaller (Fig. 1c) and have a delay in flowering time, with a more profound effect under SD (± 16 d; Fig. 2a; Table S2) than under LD conditions (± 6 d; Fig. 2a; Table S2). When we shifted plants from SD to LD conditions at 30 DAG, a time at which plants reached competence to fully initiate flowering (Schmid et al., 2003; Torti et al., 2012), LN plants delayed the floral transition by only 2 d as demonstrated by RNA in situ hybridization using the floral marker LEAFY as a probe (Fig. 1f) and morphologically using stained sections (Fig. S1c). This indicates that LN conditions delay flowering and this effect can be partially overridden by the photoperiod pathway.

The timing of the floral transition at the SAM, as visualized by the increased size of the meristem at transition and the
appearance of flower primordia, was comparable to the bolting times we determined for plants grown on either ON or LN with, on average, 16 d difference between the two conditions under SD conditions (Fig. 1g). Similar to previous reports (Tschoep et al., 2009; Castro Marin et al., 2011; Lin & Tsay, 2017) we found that nitrate supply to plants had an effect on the plastochron length and hence on the production of new leaves, masking the delay when flowering is determined based on DTB (Table S2; Fig. S2). We therefore further determined flowering time based on DTB.

We investigated whether growth in LN also affects the juvenile-to-adult phase transition as previously suggested (Vidal et al., 2014). This suggestion was based on induction of the expression of some of the miR156 precursors by acute nitrate starvation in whole seedlings (Pant et al., 2009; Krapp et al., 2011; Liang et al., 2012). A delay in the vegetative phase change might explain, at least partially, the late-flowering phenotype of LN plants. We monitored the vegetative phase change morphologically. The number of juvenile leaves was not altered (Fig. S3a–c), demonstrating that the length of the juvenile phase does not differ between ON and LN plants. However, the levels of two miR156 precursors, MIR156A and MIR156C, were increased in LN-grown rosette samples (Fig. S3d). We therefore also analyzed abundance of SPL transcripts, which are the targets of miR156 (Rhoades et al., 2002). SPLs belong to a large family of transcription factors, several of which play a role in age-dependent

![Fig. 2](image-url)  
**Fig. 2** Nitrate regulates flowering time at the shoot apical meristem (SAM) of Arabidopsis thaliana. (a) Whereas wild-type plants are significantly later-flowering in long-day (LD) and short-day (SD) conditions when grown in N-limited soil (LN), soc1-6 plants flower at the same time, indicating that SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) is required for the regulation of nitrate-dependent flowering. (b, c) LN growth causes reduced SOC1 expression in the SAM of 30-d-old SD-grown plants shifted to LD for 3 and 7 d to induce flowering and harvested at the end of the day, as demonstrated by quantitative real-time polymerase chain reaction (qRT-PCR) (b) and by RNA *in situ* hybridization (c) using a specific SOC1 probe on longitudinal sections through apices (compare arrows in optimal N soil (ON) vs LN at 3 d after shift (DAS)). (d) The strong expression of NITRATE REDUCTASE 2 (NIA2) in the center of the SAM, demonstrated by RNA *in situ* hybridization using a specific NIA2 probe on longitudinal sections through apices of ON-grown plants (closed arrow heads in ON), indicates that nitrate assimilation can take place in the SAM. The NIA2 expression domain is smaller in LN plants (d, open arrow heads in LN). (e) Lower transcript abundance of NIA1 and NIA2 was confirmed by qRT-PCR. (f, g) Nitrate reductase activity (NR) (f) and nitrate (g) measured at the SAM were significantly reduced in LN plants. Both NR and nitrate were calculated on the basis of protein measured in the same extracts, for which no difference was found between the treatments. Error bars denote SD; the statistical significance between ON and LN was calculated using Student’s t-test: *, P < 0.5; **, P < 0.01; ***, P < 0.001. Bar, 100 μm.
developmental transitions such as the vegetative phase change and the floral induction as part of the age pathway (Xu et al., 2016). We did not detect significant changes between ON and LN plants in rosettes, except for increased levels of SPL4 in LN plants (Fig. S3e). Increased expression of SPL4 in the rosettes of LN plants is inconsistent, with a delayed vegetative phase change; whereas miR156 levels decline when plants age, SPL transcript abundances increase (Wang, 2014). Together, these results show that limiting N in a nonstressful way delays flowering time but not the vegetative phase change.

SOC1 is required for the regulation of N-dependent flowering

To determine the potential contribution of known flowering pathways to nitrate-dependent flowering, we performed flowering time analyses of mutants in components of different pathways of the flowering network and wild-type plants grown in the two N regimes (Table S2). Interestingly, within our initial set, we identified only one mutant line that behaved differently from Col-0 wild-type plants in that its flowering time did not respond to the N treatment. A mutation in the SOC1 locus caused ON and LN plants to flower at the same time in both LD and SD conditions (Fig. 2a; Table S2). This observation indicates that nitrate-dependent flowering requires a functional SOC1. Interestingly, flowering data from Castro Marin et al. (2011) had already indicated that the soc1-1 mutant (Ler background) grown on agar plates displays a reduced response to nitrate. However, as the wild-type Ler accession itself presented only a weak response to nitrate in their conditions, the authors did not conclude from their results that SOC1 contributes to nitrate-dependent regulation of flowering.

SOC1 serves as a central integrator for multiple flowering pathways in the SAM (Srikanth & Schmid, 2011) and its expression is strongly upregulated in the center of the meristems when plants are shifted from noninductive SD conditions to inductive photoperiods (LD). To further examine the role of SOC1 in nitrate-dependent flowering, we grew plants in ON and LN under SD conditions for 30 d and then shifted them to LD conditions and analyzed the expression of SOC1 and its downstream targets at the SAM by qRT-PCR analyses and RNA in situ hybridization using SOC1 as a probe (Fig. 2b,c). SOC1 expression was upregulated in response to an inductive photoperiod at a much later time point and had greatly reduced levels in LN plants, indicating that SOC1 is an essential player in nitrate-dependent flowering at the SAM.

These findings contradict with an earlier study carried out using an agar growth system (Liu et al., 2013). Here the authors demonstrated that low nitrate concentrations (1 mM nitrate and 4 mM glutamine, agar-based) induce the expression of SOC1 in rosette samples at a 10-leaf stage in a 12 h : 12 h light : dark photoperiod, and suggested that this induction is mediated by an increase in the biosynthesis of GA, causing an early-flowering phenotype (Liu et al., 2013). In addition, another study (1 mM vs 3 mM KNO3, vermiculite- or agar-based) suggested that GA signaling is involved in the late-flowering phenotype caused by high nitrate content (Gras et al., 2018). In order to discover whether the GA pathway is affected, five transcripts encoding components of the GA biosynthesis and its signaling were analyzed in SD conditions. We did not detect any differences between ON and LN plants (Fig. S4), demonstrating that nitrate does not interact with GA biosynthesis and signaling when plants have the chance to adapt to available nitrate conditions in soil.

Nitrate assimilation at the SAM is regulated by N availability

To date, published data have demonstrated that nitrate uptake in the roots can be assimilated, stored in vacuoles or transferred to different parts of the plant to foster growth (Tischner, 2001). However, no evidence has come to light on whether nitrate enters and is assimilated in the SAM. If this were to occur, it would open up the possibility that the available nitrate in the SAM can directly regulate flowering genes. In an attempt to understand whether meristematic tissue contains and can assimilate nitrate, we examined the expression of NITRATE
REDUCTASE 1 and 2 (NIA1 and NIA2) genes by RNA in situ hybridization and qRT-PCR in the SAM (Fig. 2d,e). They encode enzymes catalyzing the first step in nitrate assimilation (Krapp et al., 2014). NIA1 and NIA2 are nitrate-induced genes and belong to the primary nitrate response, with changes occurring within 30 min. This first wave of the transcriptional response to nitrate does not require de novo protein synthesis or the presence of nitrate reductase, showing that these transcripts respond directly to nitrate (Gowri et al., 1992). Using transcript-specific probes for NIA1 and NIA2 on longitudinal sections of the apex, we found that both genes are expressed in the SAM (Figs 2d, S6a, b). Transcript of NIA1 was detected in leaves, the borders of the rib (RZ) and peripheral (PZ) zones of the SAM and the axillary meristem, whereas the transcript of NIA2 was detected in the center of the SAM, stretching from L3 into the RZ, and no transcript was detected in the PZ. Most importantly, using both RNA in situ hybridization and qRT-PCR, we found that expression of both genes was greatly reduced in LN compared with ON plants (Figs 2d,e, S6a,b), indicating that nitrate is regulating NIA expression in the SAM of these plants. This was corroborated by activity measurements in excised apices grown in SD conditions for 30 d (Fig. 2f) and by measurements of nitrate, which revealed significantly reduced concentrations in the SAM of LN vs ON plants (Fig. 2g). With these findings we provide the first evidence that nitrate metabolism can take place in the SAM proper and that nitrate-dependent signaling might directly interact with processes at the SAM.

Nitrate-dependent regulation of SPL gene expression at the SAM

Sequence analysis in several plant species has identified a conserved motif, termed the nitrate-responsive cis-element (NRE), in the upstream regions of many nitrate-induced genes (Konishi & Yanagisawa, 2010). The NRE sequence has been reported to be both necessary and sufficient for nitrate induction (Konishi & Yanagisawa, 2011). In addition, Konishi & Yanagisawa (2011) found that NRE-dependent transcription is only activated by nitrate and not by other N sources. We analyzed upstream intergenic regions of core players of the flowering network for the existence of putative NRE motifs (Table S4) and identified such motifs predominantly in SPL3, SPL4 and SPL5 (Fig. 3a; Table S3), which are expressed at the SAM during the floral transition (Schmid et al., 2003). NRE motifs were not found in the upstream intergenic regions of other SPLs, such as SPL9 or SPL15, which are also described to control flowering (Hyun et al., 2016). We generated transgenic plants harboring synthetic promoters with four copies of the respective NRE motif fused to a 35S minimal promoter driving the GUS reporter gene (Konishi & Yanagisawa, 2010) (Fig. 3b). Histochemical staining of seedlings grown for 4 d on full nutrition ½ Murashige & Skoog medium (10.3 mM NH4NO3, 9.4 mM KNO3) resulted in strong stains for the positive control (NRE35S::35S::NRE1) as well as for NRE35S::35S::NRE2, weaker stains for NRE35S::35S::NRE3, NRE35S::35S::NRE4, and no stain for NRE35S::35S::NRE5 and the negative control (Fig. 3c). These results demonstrate that the NRE motifs present in the upstream intergenic regions of SPL3 and SPL5 can activate transcription.

We next analyzed SPL expression via RNA in situ hybridization in the SAM after transferring ON or LN plants from SD to inductive LD conditions. Compared with ON plants, LN plants showed a delay in the rise of SPL3 and SPL5, but not SPL4 transcript (Fig. 3d). Also, under continuous SD conditions the rise in expression of SPL5 at the SAM was delayed in LN vs ON plants (Fig. 3e). qRT-PCR analyses on apices confirmed this result (Fig. 3f), indicating that timely expression of SPL3 and SPL5 at the SAM requires a positive input by nitrate signaling. In addition, SPL4 expression was reduced at the SAM of LN plants (Fig. 3f), suggesting a tissue-specific, but presumably NRE-independent, effect of N supply on the regulation of SPL4.

NIN-LIKE PROTEIN transcription factors bind to NREs in the presence of nitrate via their RWP-RK DNA-binding domain to affect expression of downstream nitrate-responsive genes (Krapp et al., 2014). NLP7 is known as the master regulator of nitrate signaling and is controlled at a subcellular level by a nitrate-dependent nuclear retention mechanism (Marcheve et al., 2013). NLP6 function is described as partially redundant to NLP7 (Guan et al., 2017). A late-flowering phenotype has been observed for the nlp7 mutants (Castaings et al., 2009). When we grew nlp6 and nlp7 mutant plant lines on full-nutrition soil in LD and SD conditions, we found that, for both mutants, flowering time was delayed compared with wild-type plants, whereas the double nlp6 nlp7 mutant flowered significantly later than either of the single mutant lines (Fig. 4a; Table S2). Furthermore, using RNA in situ hybridization, we detected NLP6 and NLP7 expression at the SAM (Fig. 4b). These results indicate that both NLPs are candidates for the direct regulation of SPL3 and SPL5 via the NREs in their promoters, suggesting a novel mechanism by which the nitrate content in the SAM acts directly to regulate expression of flowering time genes and promote flowering.

Flowering time in LN plants under SD conditions depends on the T6P pathway

As mentioned before, in LD conditions the photoperiod pathway may partly override the negative effect of LN and lead to flowering, albeit with a delay. LN plants also eventually initiated flowering in SD conditions, when the photoperiod pathway was inactive (Fig. 5c). This implies that in SD conditions a further flower-inducing signal overrides low N. We noticed that sucrose and T6P concentrations increased in rosettes of LN wild-type plants grown under SD conditions towards the end of the growth phase (Fig. 5a). This finding is consistent with an earlier study showing that nitrate starvation affects T6P concentrations in a liquid culture with seedlings (Yadav et al., 2014). To test whether this rise in T6P overrides the effect of low N, we grew 35S::amiRTPS1 plants in LN under SD conditions. T6P concentrations increased in LN-grown 35S::amiRTPS1 plants (Fig. 5b) but the rise was delayed and T6P concentrations remained below wild-type concentrations. Strikingly, the 35S::amiRTPS1 plants never flowered (Fig. 5c; Table S2). We previously reported that constitutive expression of MIR156b in the background of 35S::
Fig. 3  Nitrate-dependent expression of SQUAMOSA PROMOTER BINDING-LIKE (SPL) genes at the shoot apical meristem (SAM) of Arabidopsis thaliana. (a) Putative nitrate-responsive elements (NREs) in the upstream regulatory regions of SPL3, SPL4 and SPL5. (b) Schematic illustration of expression cassettes consisting of four copies of the respective NREs fused to a 35S minimal promoter (minP) driving a GUS reporter gene and negative control without NRE. (c) Histological staining of the synthetic promoter-GUS lines compared with the negative control. (d) RNA in situ hybridization using specific probes for SPL3, SPL4 and SPL5 on longitudinal sections through apices of plants grown in the two nitrogen (N) regimes (optimal N soil (ON) and limited N soil (LN)) in short-day (SD) conditions for 30 d, before shifting them to long days (LD) for 3, 5 and 7 d (DAS, days after the shift). Bar, 100 μm. (e) RNA in situ hybridization using a specific probe for SPL5 on longitudinal sections through apices of plants grown in continuous SD conditions and harvested at the end of the day. (f) Transcript abundances of SPL3, SPL4 and SPL5 measured by quantitative reverse transcription polymerase change reaction in apices. Bars: (c) 1 mm; (d) 100 μm; (e) 50 μm. Error bars denote SD; the statistical significance between ON and LN was calculated using Student’s t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
amiRTPS1 plants yields plants that are unable to flower on standard soil in SD conditions (Wahl et al., 2013). Growth in LN leads to decreased expression of the SPLs in the SAM by limiting nitrate (Fig. 3d–f). It is likely that the nonflowering phenotype of LN-grown 35S::amiRTPS1 plants in SD conditions is due to a stalled T6P pathway in combination with decreased SPL expression. Taken together, our data demonstrate that floral induction in the late-flowering wild-type plants grown under nitrate-limited and SD conditions largely relies on the T6P pathway. If both pathways are impeded, flowering cannot occur, highlighting the joint importance of the nitrate signaling pathway and the T6P pathway for the onset of flowering.

Discussion

Understanding the molecular mechanisms underlying the regulation of nitrate-dependent flowering is a crucial step towards the development of alternative breeding strategies for a sustainable production of staple crops under nitrate-limited conditions. In contrast to the growth systems used in previous studies (reviewed in Lin & Tsay, 2017), we used a soil-based low-N system (Tschoep et al., 2009) in which plants are able to adapt their metabolism and growth to the reduced N supply. This allowed us to study the control of flowering time without complications due to stress or major changes in central metabolite concentrations. LN prolongs the adult vegetative phase and delays flowering of A. thaliana, but the length of the juvenile vegetative phase is not altered. As seen previously, expression of some of the miR156 precursors are increased in LN leaves (Pant et al., 2009; Krapp et al., 2011; Liang et al., 2012), although expression levels of miR156 targets do not change in response to LN, contradicting earlier assumptions (Vidal et al., 2014). However, we cannot exclude the possibility that these changes become relevant at more extreme nitrate conditions, as predicted by Lin & Tsay (2017).

We found that none of the flowering pathways originating in leaves caused the delayed flowering phenotype in LN. This includes all of the sexual organs of a plant and, as such, major changes related to the floral transition, that is the production of flowers instead of leaves, are realized at the SAM. However, some of the signals that control this transition are initiated in leaves (Srikanth & Schmid, 2011). Indications that nitrate directly acts as a signal for flowering were already found in previous studies in which supplementation of a limited nitrate medium with glutamine did not rescue the flowering phenotype (Castro Marin et al., 2011; Weber & Burow, 2018). However, evidence that nitrate signaling can directly interact with the flowering network at the SAM has not yet been reported. A recent publication describes a

Fig. 4 Master regulators of nitrate signaling are present at the shoot apical meristem (SAM) of Arabidopsis thaliana. (a) Flowering time analyses of plants mutant for NIN-LIKE PROTEIN 6 (NLP6) and NLP7 based on ‘time to bolting’ (d), determined in long-day (LD) and short-day (SD) conditions on standard full-nutrition soil. (b) RNA in situ hybridization on longitudinal sections through inflorescence apices of plants grown in LD conditions using specific probes against NLP6 and NLP7. Error bars denote SD; the statistical significance between optimal N soil (ON) and limited N soil (LN) was calculated using Student’s t-test: ***, P < 0.001.
root-borne cytokinin signal that transduces nitrate availability to the SAM within a matter of days and controls the stem cell population and hence meristem size and growth (Landrein et al., 2018). It remains an open question whether this systemic signal also contributes to the regulation of flowering as previously suggested (D’Aloia et al., 2011). Our findings demonstrate that nitrate does not necessarily need a second messenger to transduce its status to the flowering network in the SAM. The local sensing of nitrate at the SAM, as strongly suggested by our study, will allow changes in the meristematic nitrate concentration to be rapidly translated into downstream events, allowing high developmental plasticity in fluctuating environmental conditions.

We found that nitrate reaches the SAM, where it can be assimilated, as also demonstrated by the presence of NIA1 and NIA2 transcripts and nitrate reductase activity measured in excised apices. These genes, which are part of the primary transcriptional response to nitrate, are repressed in the SAM under limited nitrate availability. We also found that NLP transcription factors, which convey nitrate content information to important downstream targets within the flowering network, are present in the SAM. Both SPL3 and SPL5 carry two and five functional NRE sequences in their upstream intergenic regions, respectively. These elements are targeted by NLP6 and NLP7 upon activation and nuclear retention in the presence of nitrate (Marchive et al., 2013; Guan et al., 2017). NLP6 and NLP7 belong to a family of nine nitrate-regulated transcription factors present in the A. thaliana genome, and all nine members can potentially bind to NREs (Konishi & Yanagisawa, 2013). Currently, NLP7 is

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**Fig. 5** Nitrate-signaling and the trehalose 6-phosphate (T6P) pathway act independently to control flowering in Arabidopsis thaliana. (a–c) Sucrose and T6P concentrations were measured in rosettes of wild-type plants grown in the two nitrogen (N) regimes in short-day conditions (SD) and harvested at the end of the day (a, b). Sucrose and T6P (a) concentrations rose in plants grown in limited N soil (LN plants), whereas the concentrations stayed constant in plants grown in optimal N soil (ON plants) throughout the experiment and only appeared to be significantly changed in wild-type plants in very old, senescing plant material (compare dark gray columns and 60 vs 80 d after germination (DAG) in panel b). As previously reported, sucrose and T6P concentrations are higher and lower (b), respectively, in rosettes of nonflowering 35S::amiRTPS1 line grown in LN (c) (Wahl et al., 2013). (d) Hypothetical graph on the species-specific relationship between N abundance and its effect on plant growth and flowering time in plants. Error bars denote SD; the statistical significance was calculated using Student’s t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
considered the master regulator for nitrate signaling, conveying information of a cell’s nitrate status to metabolic and developmental processes (Castaings et al., 2009). However, other NLPs have also been described to function in distinct developmental processes (Yu et al., 2016). Given that they are expressed in almost all plant organs (Winter et al., 2007; Castaings et al., 2009; Chardin et al., 2014), it will be interesting to uncover the functional significance of each of the NLPs in the future. Although we did not identify a functional NRE in SPL4 in the seedling GUS assay, its expression is decreased at the inflorescence SAM of LN plants, suggesting a different signaling mechanism. For the time being we also cannot exclude an additional contribution of miR156, as at least MIR156A and MIR156C levels were slightly but significantly increased in leaves of LN plants at some times in our analyses. Interestingly, none of the observed changes in leaves or the SAM led to a prolongation of the juvenile vegetative phase.

Two lines of evidence support the idea that a functional SOC1, a central integrator for several flowering pathways at the SAM, is required for the nitrate response; first, soc1-6 mutant plants flower at the same time in both growth regimes and, second, SOC1 expression is severely reduced in wild-type plants under nitrate-limited conditions. SOC1 transcript increases in 35S::MIMI156 plants, arguing for an effect of the age pathway on SOC1 expression at the SAM (J. W. Wang et al., 2009). In addition, the SOC1 genomic region contains several GATC boxes, which are known target sequences of SPLs, and overexpression of a miR156-resistant version of SPL3 led to an increased expression of SOC1. We therefore postulate an indirect nitrate effect on SOC1 via transcriptional regulation of SPL3 and SPL5 through NLP6 and NLP7. Interestingly, SOC1 was also shown to feed back on SPL3-5 independently of miR156 by directly interacting with their CArG boxes (Jung et al., 2016).

In addition to the NRE motifs present in the upstream intergenic regions of SPL3 and SPL5, we found more potentially interesting NRE motifs in 5′ intergenic regions of other flowering time genes (e.g. SMZ, TOE2, TOE3 and MIR156F). However, no significant or relevant changes in expression of those genes were observed in the conditions used in our study, which is why we did not pursue them further for functional relevance. The plants grown on limited N in our study did not suffer from acute nitrate starvation. This may explain why we did not find some of the targets reported in studies that used more severe conditions. We assume that changes of some of these genes, in particular regulators of FT, become relevant only when N supply is more strongly reduced and triggers a general stress level, that is, conditions in which flowering is reported to be induced rather than delayed relative to ON. For example, in a recent study (Gras et al., 2018), SMZ and its close homolog SNZ were positively regulated by nitrate and this was associated with delayed flowering in high N. However, their data indicated they are induced by an indirect route via the GA pathway (Gras et al., 2018), which did not change in our growth system. We believe that the nine NLPs encoded by the A. thaliana genome add great flexibility for tissue and affinity specificity for the individual NRE motifs. Low affinity for NRE motifs might become relevant, for example, when nitrate rises to concentrations that exceed those to which plants can easily adapt, leading to growth retardation, including a severe delay in flowering.

Interestingly, we found that the nitrate and T6P pathways converge at the same node within the flowering network. In addition to induction of FT in leaves, the T6P pathway activates SPL3-5 expression in the SAM, acting partly via a miR156-dependent pathway and partly via a miR156-independent pathway (Wahl et al., 2013). To date we cannot tell which of the SPLs is more sensitive to nitrate or T6P. It will also be interesting to learn whether the T6P and nitrate pathways converge at other important checkpoints as well, such as shown for yeast (Wilson et al., 2007) and plants (Figueroa et al., 2016) where post-translational regulation of nitrate reductase strongly depends on the T6P signaling pathway. In this sense, residual TPS1 and T6P in the 35S::amiRTPS1 knockdown line might still be able to induce flowering in ON but no longer suffices to induce flowering in LN.

Our data reveal the crucial role of central players of the flowering time network at the SAM in the perception of soil N concentrations, which can only be overridden by an input of the T6P pathway in SD conditions or the photoperiod pathway in LD conditions. This highlights the importance of these pathways in the regulation of flowering time in response to the metabolic state of the plants. Taking this as a basis, new strategies for improving crop performance under nitrate-limited conditions are possible.

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Author contributions

VW and AS conceived and designed the experiments. JJO, AS and VW performed all experiments, except for the main part of the plant work, generation of the NRE lines and the GUS stainings (CA), gathering of RNA in situ hybridization and flowering time data for NLPs (JvD), measurements of T6P concentrations (RF), analysis of the vegetative phase change and growth parameters (MAD), and generating and verifying nlp mutant lines (AK). JJO, AS and VW analyzed the data. VW wrote the paper with contributions from JJO and AS. All authors read and commented on the manuscript before submission.
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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** N-limited *Arabidopsis thaliana* wild-type (Col-0) plants display developmental changes but no response to stress.

**Fig. S2** Leaf initiation rate *Arabidopsis thaliana* (Col-0) plants.

**Fig. S3** The vegetative phase transition is not changed in nitrate-limited *Arabidopsis thaliana* plants.

**Fig. S4** Expression analyses of relevant genes in gibberelic acid (GA) signaling in *Arabidopsis thaliana*.

**Fig. S5** Analyses of the components of the trehalose 6-phosphate (T6P), age and photoperiod pathways in *Arabidopsis thaliana*.

**Fig. S6** Expression analyses of nitrate assimilation genes at the shoot apical meristem (SAM) in *Arabidopsis thaliana*.

**Table S1** Oligonucleotides used in this study.

**Table S2** Flowering time data of experiments described in this study.

**Table S3** Analyses of upstream intergenic regions of selected flowering time gene loci.

**Table S4** List of genes associated with the regulation of flowering time analyzed for Table S3.

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