Planta (2011) 233:539–552
DOI 10.1007/s00425-010-1316-5

ORIGINAL ARTICLE

Nitrate regulates floral induction in *Arabidopsis*, acting independently of light, gibberellin and autonomous pathways

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Received: 27 August 2010 / Accepted: 4 November 2010 / Published online: 27 November 2010

Abstract The transition from vegetative growth to reproduction is a major developmental event in plants. To maximise reproductive success, its timing is determined by complex interactions between environmental cues like the photoperiod, temperature and nutrient availability and internal genetic programs. While the photoperiod- and temperature- and gibberellic acid-signalling pathways have been subjected to extensive analysis, little is known about how nutrients regulate floral induction. This is partly because nutrient supply also has large effects on vegetative growth, making it difficult to distinguish primary and secondary influences on flowering. A growth system using glutamine supplementation was established to allow nitrate to be varied without a large effect on amino acid and protein levels, or the rate of growth. Under nitrate-limiting conditions, flowering was more rapid in neutral (12/12) or short (8/16) day conditions in C24, Col-0 and La*er*. Low nitrate still accelerated flowering in late-flowering mutants impaired in the photoperiod, temperature, gibberellic acid and autonomous flowering pathways, in the *fca co-2 ga1-3* triple mutant and in the *ft-7 soc1-1* double mutant, showing that nitrate acts downstream of other known floral induction pathways. Several other abiotic stresses did not trigger flowering in *fca co-2 ga1-3*, suggesting that nitrate is not acting via general stress pathways. Low nitrate did not further accelerate flowering in long days (16/8) or in *35S::CO* lines, and did override the late-flowering phenotype of *35S::FLC* lines. We conclude that low nitrate induces flowering via a novel signalling pathway that acts downstream of, but interacts with, the known floral induction pathways.

Keywords Arabidopsis · Floral induction · Nitrate · CONSTANS · FLC

Abbreviations

N	Nitrogen
NR	Nitrate reductase
GA	Gibberellic acid
CO	CONSTANS
FLC	FLOWERING LOCUS C
FT	FLOWERING LOCUS T
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
LFY	LEAFY

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Electronic supplementary material The online version of this article (doi:10.1007/s00425-010-1316-5) contains supplementary material, which is available to authorized users.

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Introduction

Nitrogen (N) is the most important inorganic nutrient for plant growth (Marschner 1995; Miller et al. 2007). The major source of N is usually nitrate, with ammonium and amino acids also sometimes making a contribution (Miller et al. 2007). The N supply affects all levels of plant function, from metabolism through to allocation and development (Marschner 1995; Crawford 1995; Stitt and Krapp 1999; Lea and Azevedo 2006; Zhang et al. 2007; Hirel et al. 2007). At a cellular level, N regulates nitrate and ammonium uptake and reduction, N and carbon metabolism, secondary metabolism and cellular growth (Scheible et al. 1997a, 2000, 2004; Wang et al. 2000, 2003; Gutiérrez et al. 2007; Vidal and Gutiérrez 2008). N regulates developmental processes like germination (Alboresi et al. 2005), shoot–root allocation (Scheible et al. 1997b; Stitt and Krapp 1999), lateral root growth (Zhang and Forde 1998; Zhang et al. 1999, 2007; Tian et al. 2008), the timing of flowering (Klebs 1913; Dickens and van Staden 1988; Bernier et al. 1993) and senescence (Wang et al. 2000; Vanacker et al. 2006).

Studies with genotypes exhibiting low nitrate reductase (NR) activity have shown that some of the responses to N are triggered by nitrate. NR-deficient genotypes accumulate high levels of nitrate but contain low levels of amino acids and other N-containing metabolites, low protein and have low rates of growth (Scheible et al. 1997a, b; Wang et al. 2004). Nitrate induces genes required for the uptake and reduction of nitrate, ammonium assimilation, the oxidative pentose pathway, and glycolysis and organic acid metabolism (Crawford 1995; Scheible et al. 1997a, 2000; Wang et al. 2004; Gutiérrez et al. 2007), and represses phenylpropanoid metabolism (Fritz et al. 2006). Nitrate regulates also shoot–root allocation (Scheible et al. 1997b), root architecture (Vidal et al. 2010), and triggers a local stimulation of lateral root growth (Zhang and Forde 1998; Zhang et al. 1999; Tian et al. 2008). The details of the signalling pathway still need to be elucidated. High-affinity nitrate transporters might play a role in sensing nitrate (Little et al. 2005; Remans et al. 2006). Nitrate-dependent induction of *IPT3* in the roots leads to increased synthesis and export of cytokinins to the shoot (Sakakibara et al. 1998; Takei et al. 2004). In *Arabidopsis* plants induced to flower by exposure to a single 22-h-long day, cytokinins increases correlate with the successive steps of the floral transition (Corbesier et al. 2003). Transcript profiling has identified many transcription factors, protein kinases and protein phosphatases that are rapidly induced or repressed by nitrate (Wang et al. 2003; Scheible et al. 2004; Gutiérrez et al. 2007).

Other responses require metabolisation of nitrate, and are presumably triggered by metabolites lying further downstream in N metabolism, by accompanying changes in other pathways, or by changes in cellular activities that occur when the N supply increases. For example, downstream signalling regulates large sets of genes involved in cellular growth, including genes encoding components of the protein synthesis apparatus (Wang et al. 2004; Scheible et al. 2004; Gutiérrez et al. 2007). There are probably multiple mechanisms for sensing changes that occur when nitrate is assimilated. In bacteria and fungi the P_{II} protein acts as a sensor for 2-oxoglutarate and glutamate, two metabolites at the interface between N and carbon metabolism. Plants contain putative homologues to the P_{II} protein (Hsieh et al. 1998; Smith et al. 2003), which have been shown to bind 2-oxoglutarate (Kamberov et al. 1995; Smith et al. 2003; Moorhead and Smith 2003). Plants also contain a large family of glutamate receptor-like (*GLR*) genes (Kang and Turano 2003; Filleur et al. 2005; Forde and Lea 2007).

The transition from vegetative growth to flowering represents one of the most important events in the life history of a plant (Koornneef et al. 1998; Simpson et al. 1999; Mouradov et al. 2002). It is regulated by an interplay between environmental and endogenous factors, which communicates when the time of the year and/or the growth conditions are favourable for sexual reproduction and seed maturation. Genetic studies in Arabidopsis have identified several floral induction signalling pathways including the photoperiod, the vernalisation the gibberellic acid (GA) and the autonomous pathways (Fig. 1; Mouradov et al. 2002; Simpson and Dean 2002; Corbesier and Coupland 2006).

The photoperiod pathway integrates inputs from the circadian clock and light receptors, and promotes flowering in long days (Suárez-López et al. 2001; Valverde et al. 2004). CONSTANS (CO) activity leads to expression of FLOWERING LOCUS T (*FT*; Wigge et al. 2005), which promotes floral initiation. In many accessions, vernalization is a prerequisite for floral induction (Sheldon et al. 2000; Kim et al. 2009) by leading to a decrease of FLOWERING LOCUS C (*FLC*; Levy and Dean 1998). The GA-signalling pathway promotes flowering (Langridge 1957; Wilson et al. 1992; Cheng et al. 2004), and requires genes for GA synthesis like *GA1* (Sun et al. 1992) and genes involved in GA-signalling like *GAI* (Lee et al. 2002). Flowering is also promoted by the so-called autonomous pathway, which includes genes encoding components of RNA processing or histone modification complexes (Simpson and Dean 2002).

These signalling pathways interact to determine the timing of flowering. The photoperiod and GA pathways converge on a common set of floral pathway integrators, including *FT* (see above), a *SUPPRESSOR OF*

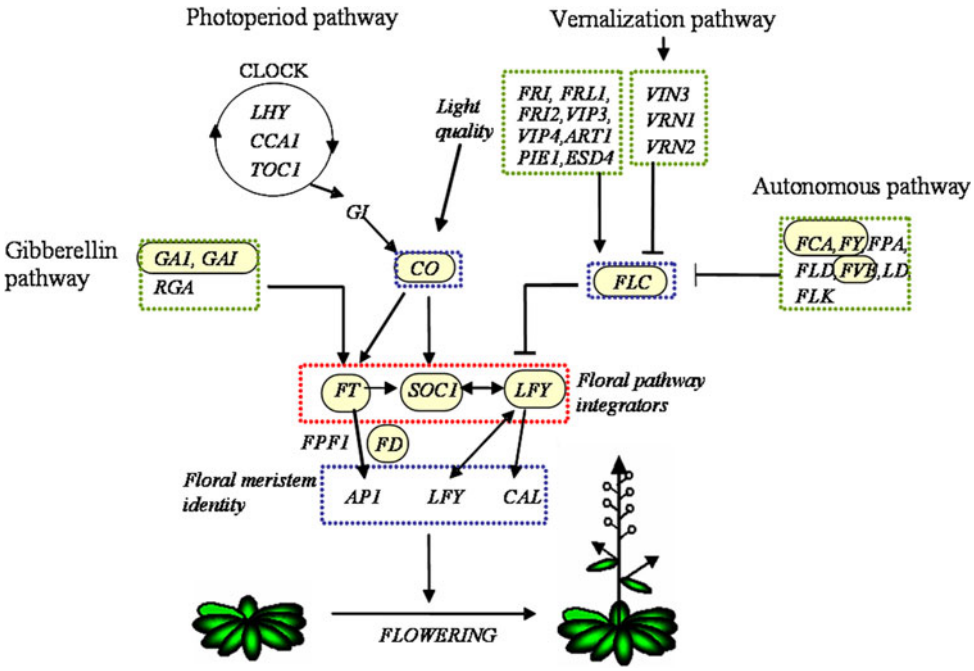


Fig. 1 Pathways controlling flowering time in *Arabidopsis*. Adapted from Corbesier and Coupland (2006)

OVEREXPRESSION OF CONSTANS 1 (*SOC1*; Lee et al. 2000; Samach et al. 2000; Moon et al. 2003) and *LEAFY* (*LFY*; Blázquez and Weigel 2000). *FLC* acts by negatively regulating *FT* and *SOC1*, and thus, overrides the photoperiod and the GA-signalling pathways. Vernalization and the autonomous pathway act by epigenetic silencing of *FLC* (Simpson and Dean 2002; Sung and Amasino 2004), allowing promotion of flowering by the photoperiod and GA pathways.

N has been known for almost a century to modify the timing of flowering (Klebs 1913). Flowering is often delayed by a high N supply and is accelerated by high rates of photosynthetic CO_2 fixation, which are thought to deplete N (reviewed in Bernier et al. 1981; Dickens and van Staden 1988; Bernier et al. 1993). It may promote reproduction and redistribution when the N supply is low, and allow exploitation of the resources to establish a larger vegetative biomass, and ultimately a larger number of seeds, when there is a more ample supply of N. The influence of N fertilisation on flowering is also an important factor in agriculture. N fertilisation has been a powerful tool to increase yield of cultivated plants, but can lead to a delay in flowering or tuberisation (Marschner 1995; Wiltshire and Cobb 1996). More generally, understanding the regulation of the plant N economy may help to improve N use efficiency (Hirel et al. 2007).

The mechanism by which the N supply influences flowering is unknown. The following experiments were carried out to address two questions. First, is the delayed transition to flowering in high N conditions triggered by changes in nitrate, or is it due to a general change in the level of organic nitrogen metabolites? Second, how does N-signalling interact with other known and genetically characterised floral induction pathways?

Materials and methods

Plant material

Landsberg erecta (La*er*), Columbia (Col-0) and C24 *Arabidopsis thaliana* accessions, different flowering-time mutants and transgenic plants with modified expression in flowering-time genes in the La*er* or Col-0 background were obtained from the Nottingham Arabidopsis Stock Centre. Transgenic lines were generated in George Coupland's group, (Max Plank Institute for Plant Breeding Research, Cologne, Germany).

Plant growth media and conditions

Seedlings were grown on horizontal agar plates under sterile growth conditions. Briefly, seeds were surface sterilized in bleach solution containing 3% NaHClO plus 0.005% (w/v) Tween 20 for 15 min, washed 3 times with sterile distilled water, resuspended in 0.15% sterile agar, and distributed on agar plates containing nutrient medium with 1, 10 or 35 mM nitrate, and 1 mM $MgSO_4$, 2.5 mM KCl, 3 mM KH_2PO_4, 2 mM $CaCl_2$, 4 mM glutamine, 1% sucrose, 0.021 mM FeEDTA, 0.075 mM H_3BO_3, 17.5 μM $MnSO_4$, 1.25 μM $ZnSO_4$, 0.75 μM $CuSO_4$, 0.5 μM $NiCl_2$, 0.375 μM Na_2MoO_4, 25 nM $CoCl_2$, and 3 mM MES (pH

5.6). Sucrose and glutamine were filtered and added after autoclaving. Some experiments used 0.5 and 10 mM nitrate without glutamine. Phosphate starvation treatments used a final KH_2PO_4 concentration of 200 μM. The plates were sealed with Leucopore tape (Beiersdorf, Hamburg, Germany), at 4°C for 3–5 days, and transferred to sterile growth chambers, with 20°C day/night and 120 μmol photons m^{-2} s^{-1} light with a day length as specified in the figure legends. Plants were visually scored each day for floral buds.

To investigate abiotic stresses, wild-type La*er* and *fcaco2ga1-3* were grown on soil. Control plants were grown in a 12 h light/12 h dark photoregime (ca. 120 μmol m^{-2} s^{-1} light), 60% relative humidity and 20°C, unless indicated otherwise. The high light treatment was 800 μmol m^{-2} s^{-1} in a 8 h light/16 h dark photoperiod, high temperature was 26°C day/22°C night in a 12 h light/12 h dark photoperiod, photochilling was 16°C day/night, 800 μmol m^{-2} s^{-1} in a 12 h light/12 h dark photoregime, and continuous light was with 120 μmol m^{-2} s^{-1} light at a temperature of 20°C.

Determination of metabolites

The soluble fraction of ethanol extracts was used to measure glucose, fructose, sucrose, nitrate and total amino acids (Fritz et al. 2006), and the residual fraction for protein and starch analysis (Geigenberger et al. 1996; Scheible et al. 1997a).

Results

Establishment of an experimental system in which nitrate and organic nitrogen can be varied independently of each other

Addition of nitrate to a plant alters the level of nitrate, the levels of downstream metabolites, and the rate of growth. Previous studies used genotypes with low or no NR activity to separate these responses. For studies of flowering, this approach has two disadvantages. First, although low-NR genotypes allow nitrate to be varied independently of downstream events, this is achieved against a background of low levels of N-containing metabolites and slow growth. To study flowering, it would be more appropriate to use a treatment that allows nitrate to be varied in the presence of high organic N, to avoid possible complications due to non-specific effects of slow growth per se. Second, it would be very tedious to screen the interaction between N and existing floral induction pathways in this genetic background, because this would require lengthy crossing programs to generate genotypes that are homozygous for multiple mutations or transgenes. For this reason, we explored whether glutamine supplementation can be used as an alternative approach. In this approach, the nitrate concentration is varied, while supplying glutamine as a constitutive source of N. Addition of 4 mM glutamine to Arabidopsis growing on 0.2 mM nitrate restored growth to above that seen on 10 mM nitrate (data not shown). Other amino acids including asparagine, aspartate, glutamate and alanine were less effective (data not shown).

Figure 2 summarizes the response when the Arabidopsis accession C24 is grown with 1, 10 or 35 mM nitrate in the presence of 4 mM glutamine as a constitutive N source on nutrient agar in weak light in a 12 h light/12 h dark photoregime. The plants were visually scored each day for the presence of a flower bud. At the time when ca. 30% of the plants in a given treatment had started to flower, all plants with a flower bud were harvested for destructive analysis. The remaining plants were visually scored for flowering for the remainder of the experiment. Decreased nitrate led to flowering at an earlier time (Fig. 2a), after producing fewer leaves (Fig. 2b) and with a lower shoot fresh weight at the time of flowering (Fig. 2c). Similar results were obtained for La*er* (see Supplemental Fig. S1) and Col-0 (see below).

Total plant fresh weight when ca. 30% of the plants had started to flower was 187, 325 and 554 mg for plants growing on 1, 10 and 35 mM nitrate (Fig. 2c, data not shown). This corresponded to day 22, 26 and 30 after germination, respectively. Based on the total plant weight and a seed weight of 0.025 mg, and assuming exponential growth over the life cycle of the plant, we estimated an average relative growth rate of about 0.24, 0.22 and 0.21 on 1, 10 and 35 mM nitrate, respectively. This provides direct evidence that inclusion of glutamine in the medium largely reverses the inhibition of growth in low nitrate. The shoot/root ratio typically decreases in low N, because root growth is inhibited less strongly than shoot growth. It is known that this response is regulated by nitrate. This decrease of the shoot/root ratio occurred when nitrate was decreased in the presence of glutamine (Fig. 2d).

Metabolites were measured in the shoot and roots as flowering commenced. Low nitrate in the medium led to a decrease of the internal nitrate pool (Fig. 2e), and a general increase of amino acids (Fig. 2f). The latter was partly due to an increase of glutamine, but analyses of the amino acid composition revealed that the levels of most other amino acids were unaltered or increased slightly in low nitrate (data not shown). There was also a small increase in the levels of glucose (Fig. 2g), fructose (not shown), sucrose (Fig. 2h) and starch (Fig. 2i), compared to plants grown on higher nitrate.

For comparison, C24 was also grown with 0.5 and 10 mM nitrate in the absence of glutamine (right hand side of the panels in Fig. 2). In these conditions, low nitrate did

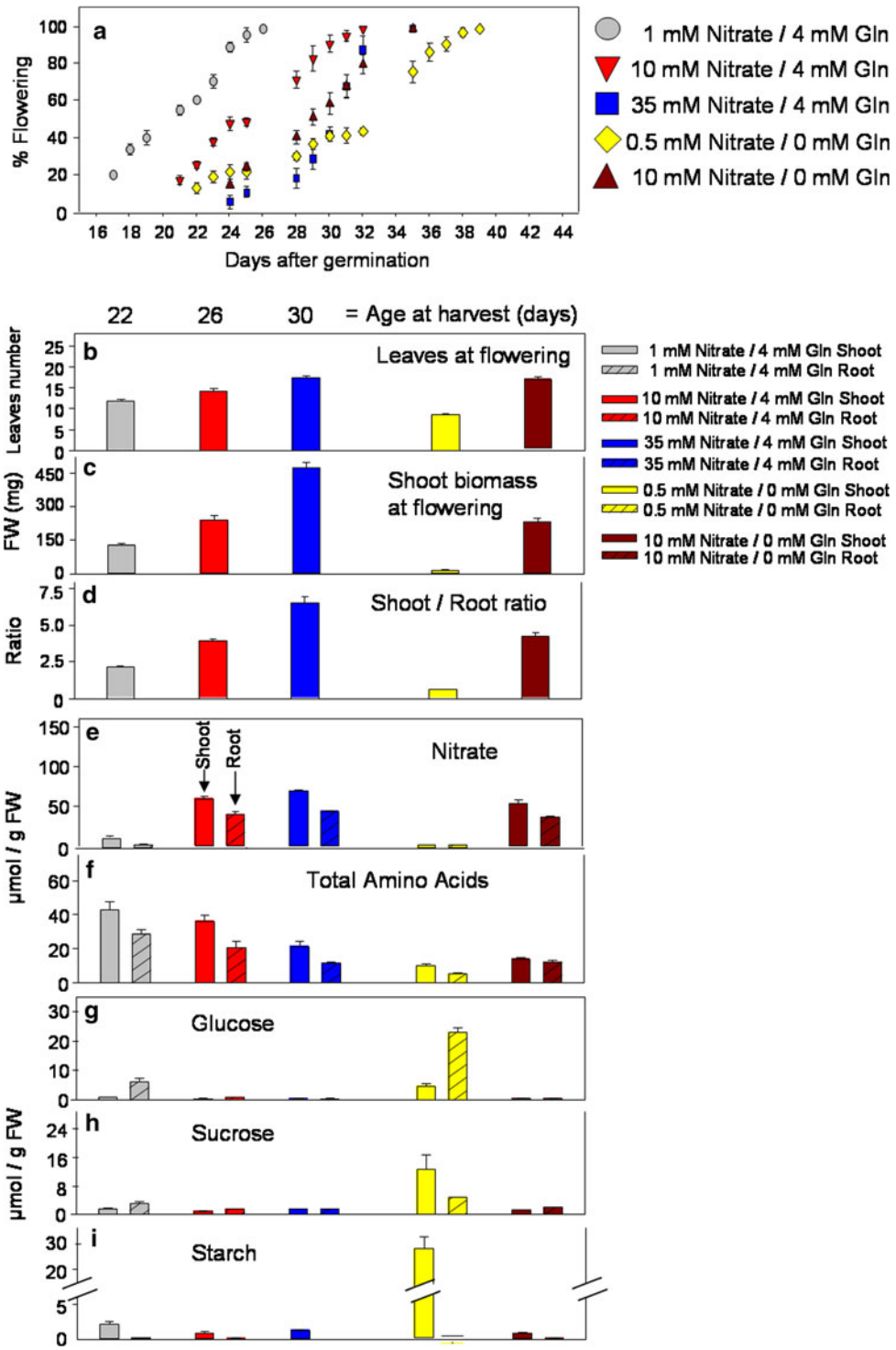


Fig. 2 Influence of nitrate on the transition to flowering and metabolite levels in the Arabidopsis accession C24 grown with glutamine as a constitutive N source. Plants were grown on medium supplemented with 4 mM glutamine and 1 (*grey*), 10 (*red*) or 35 (*blue*) mM nitrate, or on non-glutamine supplemented medium with 0.5 (*yellow*) or 10 (*brown*) mM nitrate, in a 12 h light/12 h dark photoperiod (120 µmol m^{-2} s^{-1}) at 20°C. The percentage of plants with a visible floral bud was scored each day after germination (**a**). On the day when about 30% of the plants had a visible floral bud, all plants with a floral bud were harvested. This corresponded to 22, 26 and 30 days for plants on 1, 10 and 35 mM nitrate, respectively (the plant age at harvest is noted above). Key colours corresponding to the different treatments mentioned above are similar to panel **a**, but in this case it is necessary to differentiate between shoot (*clear*) and root (*hatched*) tissues. This material was analysed to determine leaf number (**b**), shoot fresh weight (FW, **c**) and root FW (not shown), from which the shoot/root ratio (**d**) was calculated. The shoots and roots were also analysed for nitrate (**e**), total amino acids (**f**), glucose (**g**), sucrose (**h**) and starch (**i**). Metabolite levels in the shoot and root are shown as *plain* and *hatched bars*, respectively. The results are the mean ± SE of 10–15 individual plants per treatment

not lead to earlier flowering with respect to time (Fig. 2a), but it did lead to earlier flowering when this is related to leaf number or shoot weight basis (Fig. 2b, c). The acceleration of flowering by low nitrate is masked when flowering is scored on a time basis, because growth is much slower. The impact of external nitrate on internal nitrate levels was similar to that seen in the presence of glutamine (Fig. 2e). The levels of amino acids were lower than in the presence of glutamine, and decreased slightly in low nitrate. The levels of carbohydrates in low nitrate were much higher in the absence of glutamine. This is probably due to the very slow rate of growth in these conditions.

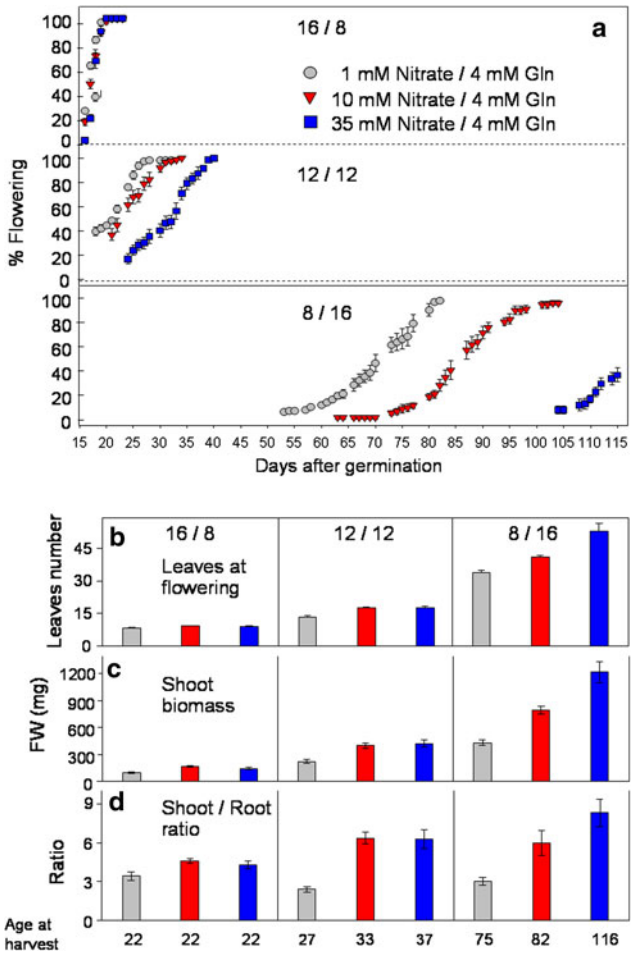


Fig. 3 Influence of nitrate on the transition to flowering and metabolite levels in the Arabidopsis accession Col-0 grown with glutamine as a constitutive N source in long, neutral and short-day conditions. Plants were grown on 1, 10 or 35 mM nitrate in the presence of 4 mM glutamine, at 20°C and a light intensity of (120 µmol m^{-2} s^{-1}) in a 16 h light/8 h dark, 12 h light/12 h dark or 8 h light/16 h dark photoregime (long, neutral and short days, respectively). The percentage of plants with a visible floral bud was scored each day after germination (**a**). On the day when about 30% of the plants had a visible floral bud, all plants with a floral bud were harvested. This was after 22 days for all plants growing in a 16/8 cycle, after 27, 33 and 37 days for plants growing in a 12/12 cycle on 1, 10 and 35 mM nitrate, respectively, and after 75, 82 and 116 days for plants growing in a 8/16 cycle in 1, 10 and 35 mM nitrate. This material was used to determine leaf number (**b**), shoot FW at the transition to flowering (**c**), the shoot/root ratio (**d**), nitrate (**e**), total amino acids (**f**), glucose (**g**), sucrose (**h**) and starch (**i**). For details and colour scheme, see the legend to Fig. 2. The results are the mean ± SE of 10–15 individual plants per treatment

Interaction of nitrate with the photoperiod

Figure 3 shows a more extensive experiment in which the accession Col-0 was grown in long (16/8), neutral (12/12) or short (8/16) day conditions in the presence of 1, 10 or 35 mM nitrate on glutamine-supplemented medium. As in the experiment of Fig. 2, when circa 30% of the plants in a given treatment had floral buds these plants were harvested for further analyses, and the remaining plants were scored visually for the remainder of the experiment. The times of harvest are noted in the legend of Fig. 3.

As expected, on high nitrate flowering occurred earlier in long-day conditions than in neutral or short-day conditions (Fig. 3a). Low nitrate did not alter the flowering time in long days, but led to earlier flowering in neutral conditions, and under short-day conditions, flowering was more rapid in nitrate-limiting conditions (Fig. 3a). Leaf number and shoot biomass at flowering were unaffected by nitrate in long days, but were decreased by low nitrate in neutral and short days (Fig. 3b, c). The shoot/root ratio decreased in low nitrate, especially in neutral and short-day conditions (Fig. 3d).

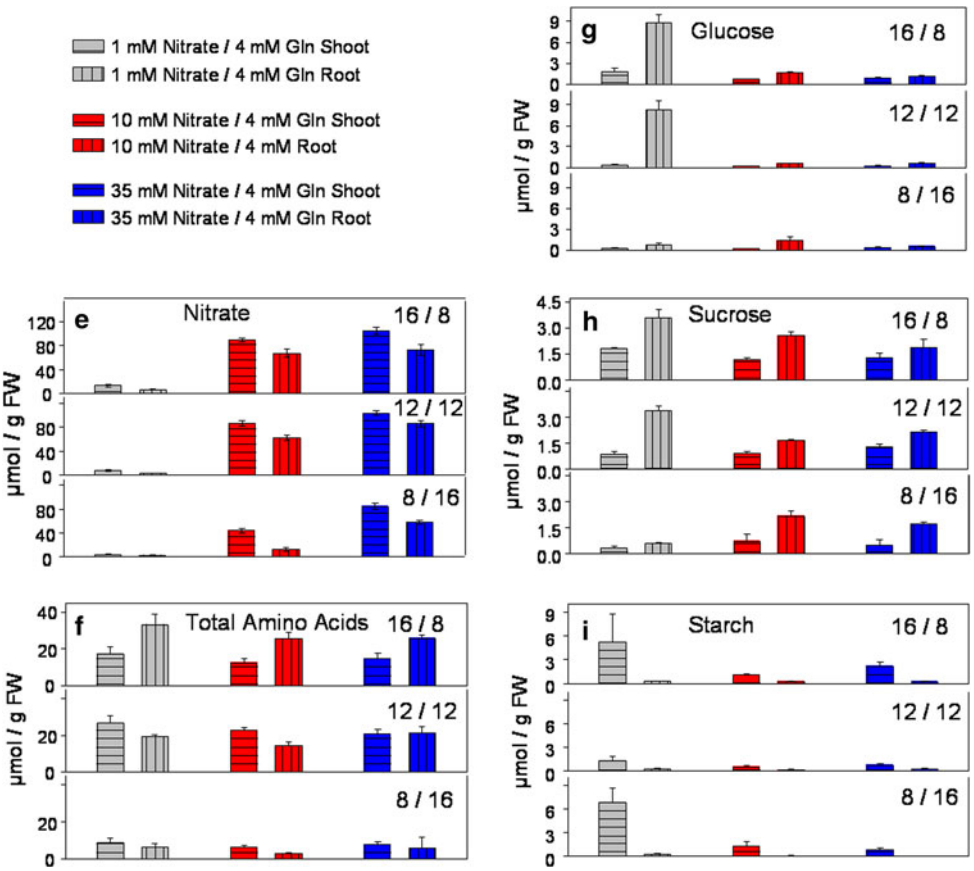
Using the procedure outlined above, we estimated that plants growing on 1, 10 and 35 mM nitrate had average relative growth rates of 0.21, 0.24 and 0.23 in a 16/8 cycle, 0.21, 0.18 and 0.17 in a 12/12 cycle, and 0.085, 0.082 and 0.063 in a 8/16 cycle. Thus, inclusion of glutamine in the medium reverses the inhibition of growth of Col-0 in low nitrate, as already seen for C24.

Analyses of plants harvested when flowering was just starting in each treatment showed that internal nitrate fell to low levels in the 1 mM nitrate treatment in all of the photoperiod regimes, and remained high in the 10 and 35 mM nitrate treatments in all three photoperiod regimes (Fig. 3e). The differential response of flowering to nitrate in the three photoperiods is therefore not due to day-length-dependent changes of internal nitrate. The trend to slightly increased levels of amino acids (Fig. 3f) and increased levels of carbohydrates (Fig. 3g–i) in low nitrate noted for C24 is confirmed in Col-0. This trend is seen in all three photoregimes. As expected, carbohydrates were lower in short days than in neutral or long-day conditions. Amino acids were also lower; this may be due to a restriction on nitrogen metabolism due to a lower supply of carbon in short-day conditions (Matt et al. 1998).

Influence of nitrate on flowering in mutants in known floral induction pathways

Figure 4 summarizes a set of experiments with mutants and transgenic plants altered in the activity of the autonomous, GA, photoperiod or vernalization floral induction pathways, and with transgenic plants with lesions that affect the floral integrators. All these experiments were carried out in a 12 h light/12 h dark photoregime, with plants growing on nutrient medium supplemented with 4 mM glutamine and provided with 1, 10 or 35 mM nitrate. Some of the mutants are in the La*er* background, and some in the Col-0 background (see legend). Both wild types show a weak response to nitrate in these conditions. The complete data set is

Fig. 3 continued



provided in the supplemental material (Supplemental Table S1).

As expected, flowering was delayed in mutants in the autonomous pathway (*fwa-1*, *fve-1*, *fy-1*) (Fig. 4a, see Supplemental Fig. S2 for more data) and a *CONSTANS* knock-out mutant (*co-2 tt4*) (Fig. 4c, see also Fig. 5c) compared to the corresponding wild type. Mutants in the GA pathway (*gai*, *ga1-3*) (Fig. 4b, see Supplemental Fig. S3 for more data) only showed a tendency. The *co-2* mutant that was used carries the *transparent testa4* (*tt4*) mutation (Putterill et al. 1995; Onouchi et al. 2000). This causes a white seed colour phenotype and was used as a visible marker to indicate the presence of the *co2* mutation, but should not affect flowering time. These mutants showed a clear tendency to flower more rapid in low nitrate. This response was retained but not strengthened in the GA-pathway mutants. The response to nitrate was significant in the autonomous pathway mutants *fwa-1*, *fy-1*, where it was more marked than in the corresponding wild type.

The triple mutant *fca1 co-2 ga1-3* is compromised in photoperiod, autonomous and GA-dependent flowering, and does not flower under either short- or long-day conditions (Reeves and Coupland 2001). No floral buds were seen when this triple mutant was grown for up to 90 days in the presence of 35 mM nitrate (Figs. 4d, 6). Strikingly, flowering occurred after ca. 75 and 50 when nitrate was decreased to 10 and 1 mM, respectively.

The photoperiod, GA and autonomous pathways act by inducing floral integrators like *FT*, *FD, SOC1*, *TFL* and *LFY* (Fig. 1). *FD* and *SOC1* have been implicated in the signalling pathway downstream of *FT*, while *TFL* is thought to antagonise *LFY*. Low nitrate accelerated flowering in *ft-7, fd-1*, *lfy*, and *tfl-1* single mutants (Figs. 4e, 5). The only exception was *soc1-1*, where flowering was hardly delayed. The latter experiment was repeated three times (Figs. 4e, 5). Flowering was strongly delayed in the *ft-7 soc1-1* double mutant (Figs. 4e, 5). The response to nitrate was qualitatively stronger after repressing some of these floral integrators, including *ft-7* single mutant and the *ft-7 soc1-1* double mutant. These results suggest low nitrate promotes flowering via a mechanism that operates independently of the photoperiod, GA and autonomous floral induction pathways, and that enters downstream of the known floral integrators.

The finding that the response to nitrate is stronger in *co-2 tt4*, the *ft-7 soc1-1* double mutant and the *fca1 co-2 ga1-3* triple mutant could be explained if nitrate acts in

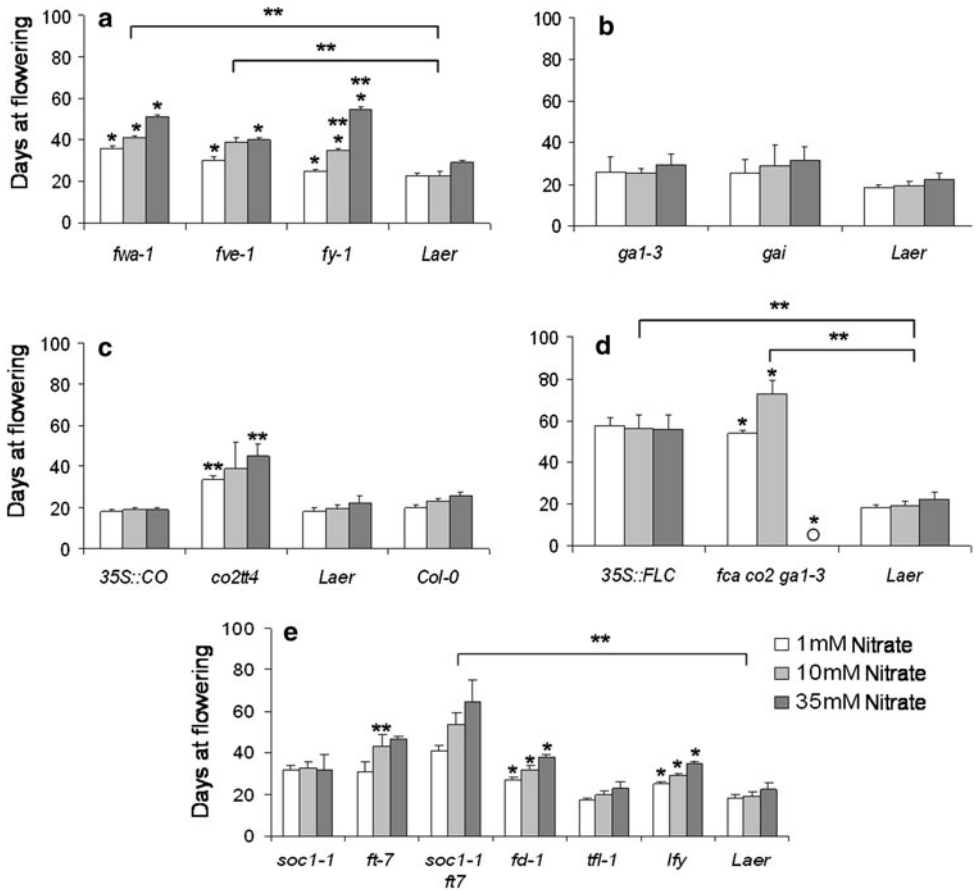


Fig. 4 Summary of the response of flowering to nitrate in mutants in the floral induction pathways and the floral integrators. The percentage of plants with a visible floral bud was scored each day after germination and, in order to get an overview of the flowering time for the different genotypes, the time at which 30% of the plants induced to flower is represented as the mean ± SD. All genotypes were grown on 1, 10 or 35 mM nitrate in the presence of 4 mM glutamine as a constitutive N source in a 12 h light/12 h dark (ca. 120 µmol m^{-2} s^{-1}) at 20°C. **a** Autonomous pathway (*fwa-1*, *fve-1*, *fy-1*; see supplemental Fig. S2 for the original data), **b** gibberellic acid pathway (*gai*, *ga1-3*; see Supplemental Fig. S3 for the original data), **c** photoperiod pathway (*35S::CO, co2 tt4*; see Fig. 5 for the original data), **d** constitutive overexpression of *FLC* (*35S::FLC*), **e** the *fca co2 ga1-1* triple mutant (see Fig. 6) and floral integrators lying downstream or *CO* and *FLC* (*ft-7, soc1-1*, *ft-7 soc1-1*, *fd-1*, *lfy*, *tfl-1*; see Fig. 5; supplemental Fig. S2 for the original data). The wild-type background was La*er* for all the mutants. The response in wild-type La*er* and wild-type Col-0 is shown for comparison. All experiments were performed with 11–22 individual plants per treatment. *Open circle* means that no plant showed floral bud in that condition. Response to different nitrate concentrations was analysed for each genotype (*$P < 0.05$, determined with one-way ANOVA for independent samples). **Pairwise significant differences between means (relative to wild-type La*er*) determined with one-way ANOVA followed by a multiple comparison test (Tukey's honestly significant difference test)

parallel with other signalling pathways, and its impact can be more clearly detected when their pathways are inhibited. In this case, constitutive activation of other floral induction pathways should override the effect of low nitrate. As already noted, nitrate has a strong impact on flowering time in short days, but little effect in long days when the photoperiod pathway is activated. This indicates that nitrate acts in parallel to and is redundant with the photoperiod pathway. Flowering is also independent of the nitrate supply in *35S::CO* mutants, where the photoperiod pathway is constitutively activated (Fig. 5b). The autonomous pathway and the vernalisation pathways both act by repressing *FLC* (Fig. 1). The interaction of nitrate with these pathways was investigated in *35S::FLC* mutants, which have constitutive overexpression of *FLC* and strongly delayed flowering. The response to nitrate was abolished in *35S::FLC* mutants (Fig. 4d).

Summarizing, the promotion of flowering by low nitrate becomes more marked when the other signalling pathways are attenuated or inhibited, but is blocked by constitutive overexpression of *FLC*. These observations are consistent with the idea that nitrate modulates flowering time via a pathway that acts in parallel with the autonomous,

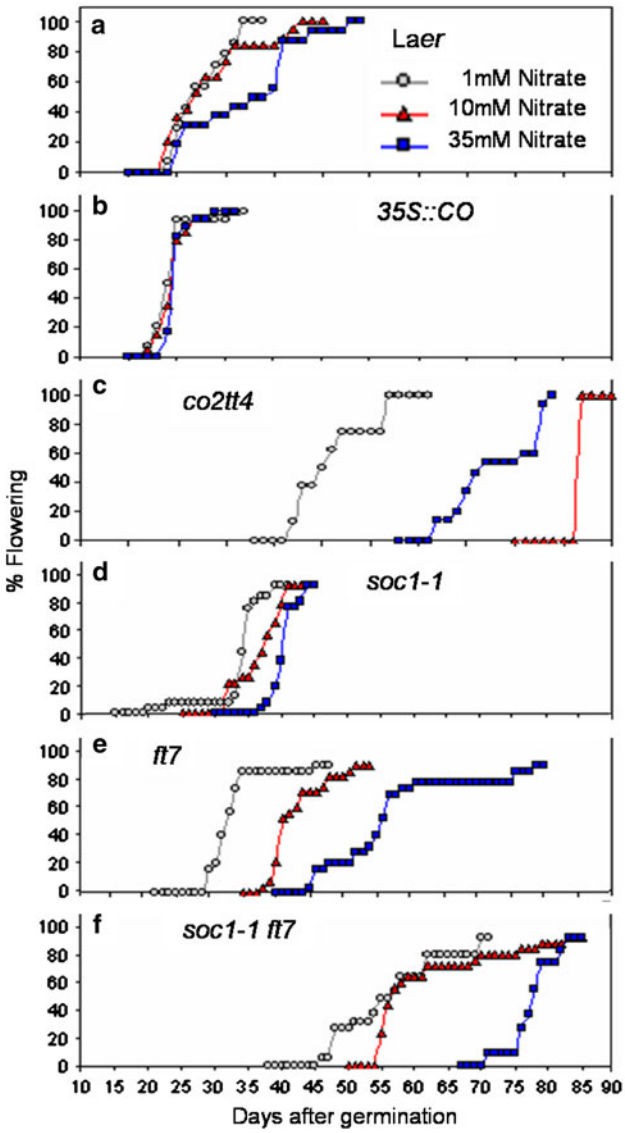


Fig. 5 Response to nitrate in mutants in the photoperiod pathway, and in floral integrators lying downstream of this pathway. All genotypes were grown on 1, 10 or 35 mM nitrate in the presence of 4 mM glutamine as a constitutive N source in a 12 h light/12 h dark (ca. 120 µmol m^{-2} s^{-1}) at 20°C. **a** La*er* wild-type, **b** *35S::CO*, **c** *co2 tt4*, **d** *soc1-1*, **e** *ft-7* and **f** *ft-7 soc1-1* double mutant. All experiments were performed with 11–22 individual plants per treatment. For colour scheme, see Fig. 2

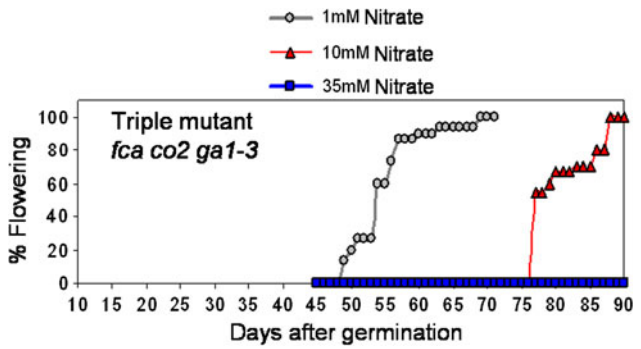


Fig. 6 Response to nitrate in the *fca co2 ga1-3* triple mutant. The triple mutant was grown on 1 (*grey circle*), 10 (*red triangle*) or 35 (*blue square*) mM nitrate in the presence of 4 mM glutamine as a constitutive N source in a 12 h light/12 h dark (ca. 120 µmol m^{-2} s^{-1}) at 20°C. 12–15 individual plants were used per treatment. The response in wild-type La*er* is shown in Fig. 5a. The experiment was performed three times with similar results. For colour scheme, see Fig. 2

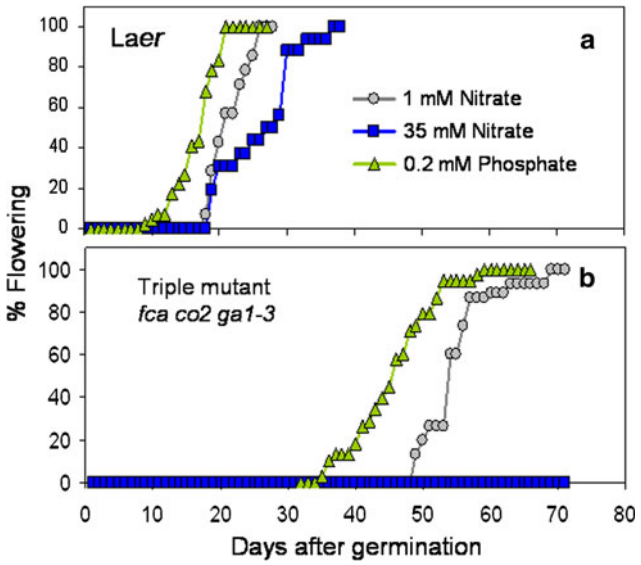


Fig. 7 Response of flowering time to low phosphate. **a** La*er,* wild type grown on 1 mM KNO_3 ($n = 14$), 35 mM KNO_3 ($n = 22$) or 0.2 mM KH_2PO_4 ($n = 41$). **b** *fca co2 ga1-3* triple mutant on 1 mM KNO_3 ($n = 15$), 35 mM KNO_3 ($n = 14$) or 0.2 mM KH_2PO_4 ($n = 48$). Treatments of 1 mM and 35 mM KNO_3 contained 3 mM phosphate, so that, were used for comparison to the 0.2 mM phosphate treatment. All mediums were supplemented with 4 mM glutamine. Plants were grown in a 12 h light/12 h dark photoperiod at 20° C day/night and 12 h of light period

photoperiod and GA pathways, that enters downstream of the known floral integrators, but is repressed by *FLC*.

Response to low phosphate

To investigate whether other nutrients modulate flowering via a similar mechanism to nitrate, wild-type La*er* and the *fca1 co-2 ga1-3* triple mutant were grown in the presence of excess (3 mM) and limiting (0.2 mM) phosphate (Fig. 7). Low phosphate led to earlier flowering in wild-type La*er* (Fig. 7a). Flowering in the triple mutant was suppressed for at least 75 days in high phosphate (plants never flowered), but was initiated after 30–50 days in 0.2 mM phosphate (Fig. 7b).

Response to abiotic stresses

General stress can also lead to early flowering. To provide further evidence that low nitrate does not act via a general stress pathway, we investigated whether abiotic stresses can trigger flowering in the *fca1 co-2 ga1-3* triple mutant (Fig. 8). High light (Fig. 8a), high temperature (Fig. 8b), photochilling (Fig. 8c) and continuous light treatments (Fig. 8d) lead to earlier flowering in wild-type La*er*. Except for photochilling, the effect was similar to or larger than the effect of nitrate in wild-type plants (see e.g., Fig. 5a). None of these treatments induced flowering in the triple mutant.

Discussion

The experiments in this paper were carried out to investigate how nitrate regulates flowering, by exploiting genetic tools available in Arabidopsis. N deficiency often induces early flowering (Klebs 1913; Dickens and van Staden 1988; Bernier et al. 1993). Limiting N leads to many changes in the plant including the depletion of nitrate, ammonium and amino acids, decreased protein, accumulation of carbohydrates especially starch, decreased growth, a change in allocation to favour root growth relative to shoot growth, and early senescence of old leaves. To separate the specific effects of nitrate from more general changes due N deficiency, we used glutamine as a constitutive N supply, and varied the nitrate in the medium. Plants growing on 1 mM nitrate contained low internal nitrate, but showed similar rates of growth to plants in 10 and 35 mM nitrate, and had slightly elevated amino acid levels. This shows that glutamine can efficiently replace nitrate as an N source.

Growth in low N typically leads to a decrease of the shoot:root ratio (Marschner 1995). This decrease is partly due to nitrate signalling. Thus, the shoot:root ratio is high in low-NR mutants where organic N is low but nitrate is high (Scheible et al. 1997b), and lateral root growth is directly regulated by nitrate (Zhang and Forde 1998; Zhang et al. 1999; Tian et al. 2008). The decrease of the shoot:root ratio in our experiments provides evidence that nitrate signalling is operating in our growth system.

Plants grown on low nitrate flowered earlier than plants grown on high nitrate in this glutamine-supplemented system. Flowering was accelerated, irrespective of whether it was scored relative to time, leaf number or shoot fresh weight. This acceleration was observed in three different accessions (Col-0, La*er*, C24). When C24 (data not shown) or the other accessions were grown on low and high nitrate in the absence of glutamine supplementation, a more complicated response was found. While low nitrate still led to earlier flowering on a leaf number or shoot fresh weight

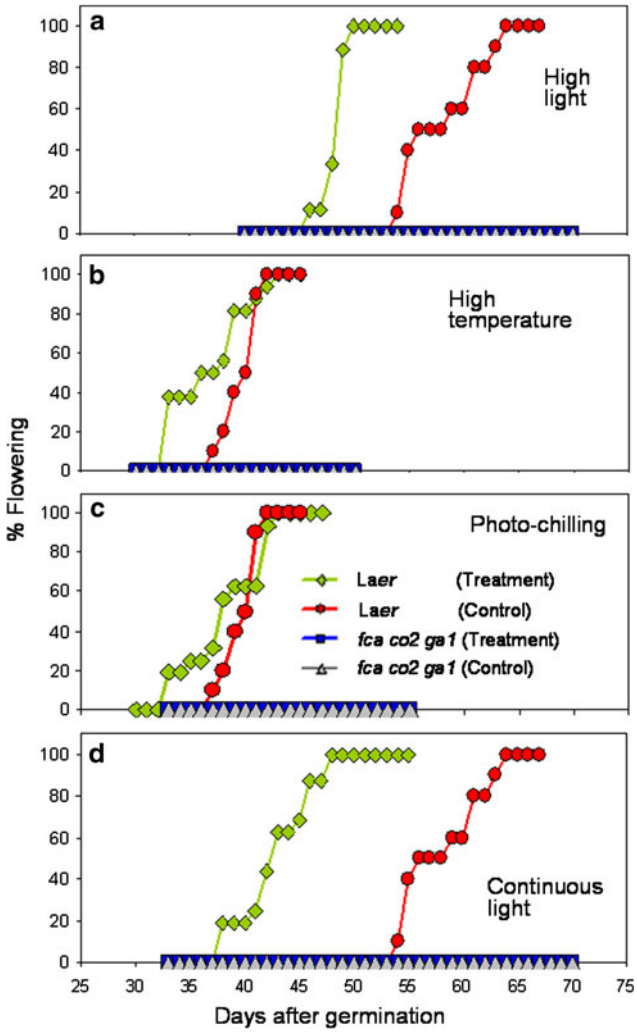


Fig. 8 Response of flowering time to abiotic stress in wild-type La*er* and the *fca co2 ga1-3*, triple mutant. Controls were grown in a 12 h light/12 h dark photoregime (ca. 120 µmol m^{-2} s^{-1}) and 20°C, unless stated otherwise. **a** High light. *Treatment* 800 µmol m^{-2} s^{-1} in an 8 h light/16 h dark photoregime. *Control* ca. 120 µmol m^{-2} s^{-1}. La*er* high light, $n = 16$; La*er* control, $n = 10$; *fcaco2ga1-3* high light, $n = 16$; *fcaco2ga1-3* control, $n = 16$. **b** High temperature. *Treatment* 26°C day/22°C night, 12 h light/12 h dark; 120 µmol m^{-2} s^{-1}. *Control* 20°C day/20°C night, 12 h light/12 h dark; 120 µmol m^{-2} s^{-1}. La*er* high temperature, $n = 16$; La*er* control, $n = 10$; *fcaco2ga1-3* high temperature, $n = 16$; *fcaco2ga1-3* control, $n = 16$. **c** Photochilling. *Treatment* 16°C day and night; 800 µmol m^{-2} s^{-1}, 12 h light/12 h dark photoregime. *Control* 20°C day/20°C night, 12 h light/12 h dark; 120 µmol m^{-2} s^{-1}. La*er* photochilling, $n = 16$; La*er* control, $n = 10$; *fcaco2ga1-3* photochilling, $n = 16$; *fcaco2ga1-3* control, $n = 16$. **d** Continuous light. *Treatment* continuous light, 120 µmol m^{-2} s^{-1}, 20°C. *Control* 20°C day/20°C night, 8 h light/16 h dark; 120 µmol m^{-2} s^{-1}. La*er* continuous light, $n = 16$; La*er* control, $n = 10$; *fcaco2ga1-3* continuous light, $n = 16$; *fcaco2ga1-3* control, $n = 16$

basis, flowering was slightly delayed in time. This is probably due to the very slow growth of the plants. Measurements of metabolites in plants grown in the glutamine-supplemented growth system confirmed that the internal

nitrate decreased to low levels in the low nitrate treatment, while amino acids remained high. This provides evidence that the signal that regulates flowering is related to nitrate itself, or a metabolite that is formed from nitrate but lies upstream of glutamine. At least in these conditions, amino acids do not appear to serve as controlling factors of the floral transition (Corbesier et al. 1998, 2001; Suárez-López et al. 2001).

Several floral induction signalling pathways have been genetically characterised, including the photoperiod, the temperature, the gibberellin and the autonomous pathways. Mutants and transformants that are attenuated or disrupted in these signalling pathways still showed an acceleration of flowering in low nitrate. There was an especially strong response in the *fca-1 co-2 ga1-3* triple mutant, which is blocked in the photoperiod, autonomous and gibberellin floral signalling pathways. This triple mutant, which usually does not flower (Reeves and Coupland 2001), flowered after 45–50 days in low nitrate. The photoperiod and GA pathways converge on a common set of floral pathway integrators, including *FT*, *SOC1* and *LFY* (Blázquez and Weigel 2000; Lee et al. 2000; Samach et al. 2000; Moon et al. 2003; Boss et al. 2004). Mutants in *ft7* and *lfy* as well as the *ft7 soc1-1* double mutant still showed an acceleration of flowering in low nitrate. These results indicate that nitrate acts by a separate signalling pathway, which enters downstream of the photoperiod, temperature, autonomous and vernalisation signalling pathways.

Although nitrate appears to act via a separate pathway, it interacts with other floral induction pathways. Nitrate has no detectable effect in long days or in *CO* overexpressing lines where photoperiod signalling is strongly activated. It has a particularly marked effect on flowering in short days or in *co2* and *ft* mutants where photoperiod signalling is weakened or abolished. The vernalisation and autonomous pathways act by epigenetic silencing of *FLC* (Simpson and Dean 2002; Sung and Amasino 2004; Alexandre and Hennig 2008), which itself acts as a negative regulator of *FT* and *SOC1*. Correspondingly, overexpression of *FLC* overrides the photoperiod, autonomous and gibberellin floral induction pathways. Constitutive overexpression of *FLC* prevented early flowering in low nitrate. This implies that overexpression of *FLC* from the *CaMV 35S* promoter inhibits unknown components in the nitrate-floral signalling pathway. Interestingly, low nitrate still induced earlier flowering in autonomous pathway mutants (see above), even though such mutants contain elevated *FLC* mRNA levels (Michaels and Amasino 1999; Sheldon et al. 2000; Simpson and Dean 2002, Simpson et al. 2004). This difference might be due to the much higher levels of *FLC* expression in *35S::FLC* plants, or to differences in the spatial patterns of expression of *FLC* expression between *35S::FLC* plants and autonomous pathway mutants. An alternative explanation is that nitrate is able to repress *FLC* when this floral repressor is expressed from its own promoter but not when it is constitutively and strongly expressed from the *CaMV 35S* promoter. However, this seems unlikely to be the sole explanation, because *FLC* is thought to act by repressing floral integrators like *FT*, *SOC1* and *LFY*, and the response of flowering to nitrate is retained and even accentuated in *lfy* and *ft-7* mutants and in *ft-7 soc1-1* double mutants. There is also no evidence that low nitrate decreases *FLC* transcript levels (Scheible et al. 2004; data not shown). Therefore, we can conclude that the effect of nitrate is dependent on *FLC* levels and perhaps there are some ecotypes (as C24) with an extreme vernalisation requirement that should be insensitive to nitrate levels (with respect to flowering time).

Recently, evidence has been adduced that further pathways operate to induce flowering in response to stress, including a salicylic acid that leads to early flowering under UV-C stress and interacts with *FLC*, *FT* and some components of the autonomous pathway (Martinez et al. 2004; Wada et al. 2010) and a high-temperature pathway that involves some components of the autonomous pathway (Blázquez et al. 2003; Balasubramanian et al. 2006). Several lines of evidence indicate that low nitrate does not act via a general stress pathway. First, nitrate accelerated flowering, even when plants were provided with glutamine and had high levels of amino acids and protein, and rates of growth comparable to those on high nitrate. Second, nitrate still led to early flowering in the *fca1co-2ga1-3* triple mutant, whereas the acceleration of flowering by several stresses including high temperatures was blocked in this triple mutant. Third, regulation of flowering by salicylic acid pathway is blocked by mutations in the autonomous pathway including *fve-3* and *fca-9*, and probably required increase expression of *FT* (Martinez et al. 2004), whereas low nitrate still induced flowering in *fve* and *fca* mutants, and in the *ft-7* mutant.

Early flowering in low nutrient conditions provides an escape strategy, by promoting seed set and reproduction when the nutrient supply is exhausted. This may be important for a weed like Arabidopsis. Functionally, it will be closely linked with leaf senescence, to remobilise nutrients from vegetative tissues into seeds. The interaction between nitrate and the photoperiod pathway and other FLC-modulated pathways uncovered in our laboratory experiments is consistent with the idea that the nitrate-signalling pathway provides an escape strategy. Thus, photoperiod signalling leads to flowering in long days and allows flowering to be adjusted to the season, irrespective of the N supply. However, if the N supply is low, Arabidopsis flowers earlier, even in short days. Similarly, in accessions with a biennial life history, vernalisation is required to inactivate *FLC* and allow flowering in the

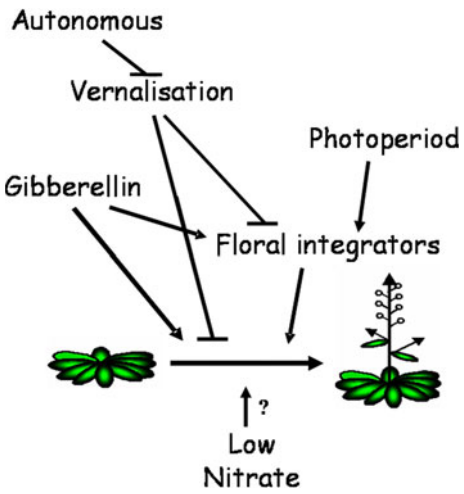


Fig. 9 Schematic model of flowering pathways in *Arabidopsis*. The nitrate-signalling pathway acts in parallel to and enters downstream of the photoperiod, GA and autonomous pathways and known floral integrators, but can be overridden by these pathways

following spring. The observation that FLC strongly inhibits flowering even in low N indicates that this strategy may be fairly resilient to changes in N, although studies in biennial accessions are needed to test this prediction.

Summarizing, our results lead to the proposal that nitrate modulates flowering time via a pathway that acts in parallel with the autonomous, photoperiod and gibberellic acid floral induction pathways, enters downstream of the known floral integrators, but is repressed by *FLC* (see schematic model for flowering pathways in *Arabidopsis*, Fig. 9). Further elucidation of nitrate-dependent signalling pathway will require the development of an experimental system where it is possible to change N and generate a synchronized transition to flowering, in order to analyse changes of transcripts and proteins at the shoot apex.

Acknowledgments The work was supported by the Max-Planck-Society and by the German Ministry for Research and Technology (BMBF) funding the project GABI Verbund Arabidopsis III 'Gauntlets, 'C and Nutrient Signaling: Test Systems, and Metabolite and Transcript Profiles' (0312277A).

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