

# Additional targets of the *Arabidopsis* autonomous pathway members, FCA and FY

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## Abstract

**A central player in the *Arabidopsis* floral transition is the floral repressor *FLC*, the MADS-box transcriptional regulator that inhibits the activity of genes required to switch the meristem from vegetative to floral development. One of the many pathways that regulate *FLC* expression is the autonomous promotion pathway composed of FCA, FY, FLD, FPA, FVE, LD, and FLK. Rather than a hierarchical set of activities the autonomous promotion pathway comprises sub-pathways of genes with different biochemical functions that all share *FLC* as a target. One sub-pathway involves FCA and FY, which interact to regulate RNA processing of *FLC*. Several of the identified components (FY, FVE, and FLD) are homologous to yeast and mammalian proteins with rather generic roles in gene regulation. So why do mutations in these genes specifically show a late-flowering phenotype in *Arabidopsis*? One reason, found during the analysis of *fy* alleles, is that the mutant alleles identified in flowering screens can be hypomorphic, they still have partial function. A broader role for the autonomous promotion pathway is supported by a microarray analysis which has identified genes mis-regulated in *fca* mutants, and whose expression is also altered in *fy* mutants.**

**Key words:** *Arabidopsis*, autonomous promotion pathway, FCA, FLC, flowering time, FY, microarray, polyadenylation, RNA processing, vernalization.

## Introduction

The switch to flowering is a major developmental transition in the plant life cycle (Simpson and Dean, 2002). Plants undergo an initial period of vegetative growth and later in

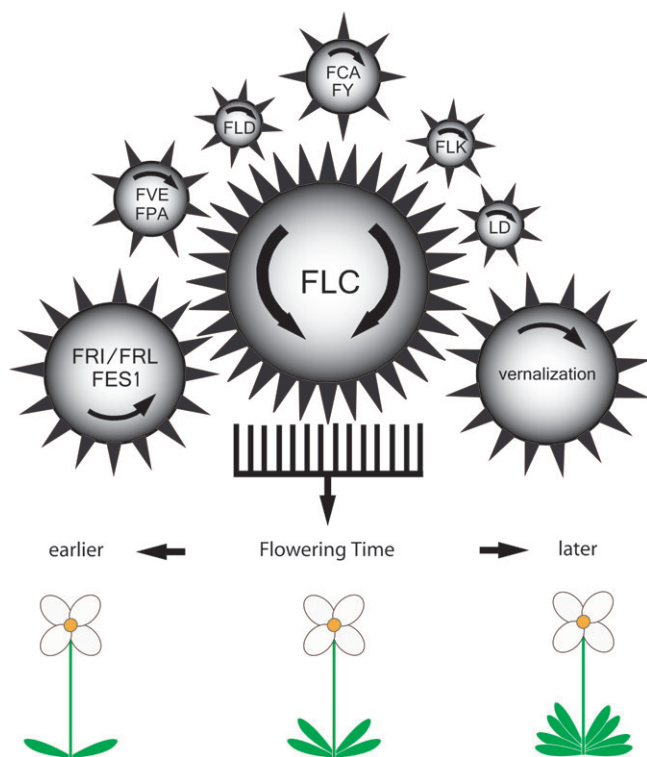
development the meristem undergoes a change in fate and enters reproductive development, producing flowers and differentiating the germ line. The timing of this transition is crucial for reproductive success as maximal pollination and seed set occur when environmental conditions are most favourable. Thus, great variability in flowering time has evolved in different plant species and varieties as they have adapted to growth in different conditions. In order to achieve this tight regulation of flowering, multiple pathways have evolved to integrate several environmental and endogenous cues (Battey, 2000; Bernier, 1988). These were elegantly dissected using a physiological approach in a range of plant species, and notable in this respect was the work on photoperiod response analysed in *Sinapis alba* by Bernier and colleagues (Bernier, 1988; Bernier *et al.*, 1993).

The genetic dissection of flowering time control was greatly facilitated by the focus on *Arabidopsis thaliana* by many plant scientists. Flowering research in *Arabidopsis* started in several laboratories in the 1950s, where the focus was on natural variation (Napp-Zinn, 1955), although some mutagenesis was undertaken (Relichová, 1976). However, the induced mutations that have impacted significantly on progress to date were developed by Maarten Koornneef and colleagues in Wageningen (Koornneef *et al.*, 1991). His group identified a series of late-flowering mutations from the rapid-cycling Landsberg *erecta* parent. Development of map-based gene cloning tools, insertional mutagenesis systems, facile transformation, well-characterized natural variants, extensive mutant collections, and now the full genome sequence have greatly accelerated the dissection of flowering time control in *Arabidopsis thaliana*. There is now a framework for the regulatory gene hierarchy controlling flowering (Simpson *et al.*, 1999; Boss *et al.*, 2004) and ideas of how it might differ between varieties and species (Simpson and Dean, 2002).

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A central regulator in this hierarchy is the floral repressor *FLOWERING LOCUS C* (*FLC*) which, together with other floral repressors *TERMINAL FLOWER1* (*TFL1*), *TERMINAL FLOWER2* (*TFL2*), *SHORT VEGETATIVE PHASE* (*SVP*), and *TARGET OF EAT1/2* (*TOE1/2*), delays the transition to flowering (Bradley *et al.*, 1997; Sheldon *et al.*, 1999; Hartmann *et al.*, 2000; Gaudin *et al.*, 2001; Aukerman and Sakai, 2003). There has been a great deal of focus on regulation of the expression of the *FLC* repressor which is regulated by a number of independent pathways (Baurle and Dean, 2006; Henderson and Dean, 2004) (Fig. 1). It is down-regulated by vernalization (through genes *VIN3* (Sung and Amasino, 2004), *VRN2* (Gendall *et al.*, 2001) and *VRN1* (Levy *et al.*, 2002)) and a series of proteins classified as the autonomous promotion pathway. It is up-regulated by a number of genes, including *FRIGIDA* (*FRI*) (Johanson *et al.*, 2000), *FRIGIDA-LIKE1* (*FRL1*) (Michaels *et al.*, 2004), *EFS* (*SDS8*) (Kim *et al.*, 2005; Zhao *et al.*, 2005), *ARP6* (Choi *et al.*, 2005; Deal *et al.*, 2005; Martin-Trillo



**Fig. 1.** Cog model of interactions regulating *FLC*. Several pathways feed into *FLC*. The outcome of these inputs is a quantitative regulation of flowering time. The positive and negative regulators of *FLC* are represented as cogs that turn in opposite directions and impact *FLC* antagonistically. Vernalization and the autonomous promotion pathway genes (*FLK*, *LD*, *FY*, *FCA*, *FLD*, *FPA*, and *FVE*) are negative regulators of *FLC*, they turn the wheel towards early flowering, whereas positive regulators of *FLC* such as *FRI* together with *FRL* and *FES1* push *FLC* function towards late flowering. Whichever side has a greater combined impact on *FLC* regulation determines *FLC* levels and thus time of flowering. The pathways regulating *FLC* do not necessarily act simultaneously but rather at different times in development and in response to environmental cues.

*et al.*, 2006), and *FES1* (Schmitz *et al.*, 2005). In this paper, the focus is on one of those pathways, the autonomous promotion pathway and current understanding of it is reviewed. There is speculation on whether the autonomous promotion pathway has a more generic role than flowering time control and some microarray experiments, aimed at identifying components required for *FCA* repression of *FLC* plus targets in addition to *FLC*, are briefly summarized.

## The floral repressor, *FLC*

*FLC* is a MADS box transcriptional repressor, expressed predominantly in shoot and root apices and the vasculature. It acts to repress flowering quantitatively through blocking expression of the floral pathway integrators, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999, 2000). The mechanism by which it does so is not well understood, although a MADS-box binding site within the *SOC1* promoter is required (Hepworth *et al.*, 2002) and it has been found bound *in vivo* to regulatory sequences of *SOC1* and *FT* (Searle *et al.*, 2006). Allelic variation at *FLC* has been found to contribute to natural variation in vernalization requirement between different *Arabidopsis* accessions (Gazzani *et al.*, 2003; Michaels *et al.*, 2003; Werner *et al.*, 2005) with some of the naturally occurring weak *FLC* alleles being caused by changes in expression rather than alterations of protein function. Landsberg *erecta* (*Ler*) contains a Mutator-like transposon at the 3' end of the large *FLC* intron 1 (Gazzani *et al.*, 2003; Michaels *et al.*, 2003). This induces an RNAi-mediated suppression of at least some of the *FLC* transcripts so reducing overall *FLC* levels (Liu *et al.*, 2004).

## Repression of *FLC* by genes of the autonomous promotion pathway

Analysis of mutants (initially *Ler* but also recently *Col*) that flower very late in both long and short day photoperiods defined the autonomous promotion pathway (Koornneef *et al.*, 1991). They have subsequently all been shown to regulate the common target, *FLC*. Their late-flowering phenotype is overcome by vernalization, so vernalization and the autonomous promotion pathway are considered to function in parallel. In the absence of *FRI*, this pathway is the major activity regulating *FLC* levels and vernalization requirement (Fig. 1). There are currently seven members of the autonomous promotion pathway; *FCA*, *FY*, *FPA*, *FVE*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LATE KH MOTIF* (*FLK*), and *FLOWERING LOCUS D* (*FLD*) (Table 1). Some mutations (in *LD*, *FLD*, and possibly *FLK*) cause a late-flowering phenotype in a Columbia background but are not late or only slightly late-flowering in a *Ler* background (Lee *et al.*, 1994b; Sanda and Amasino, 1996b).

**Table 1.** Summary of the autonomous promotion pathway genes

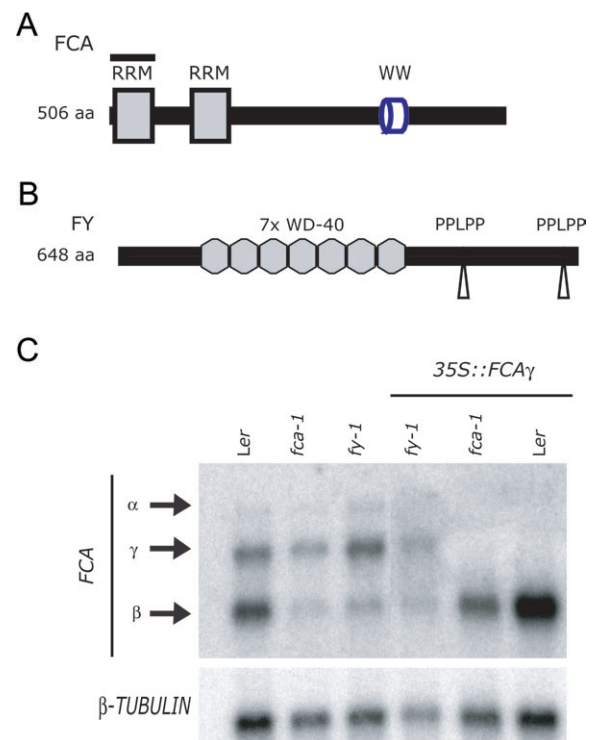
The AGI column displays the gene identifier associated to the gene name obtained from [www.arabidopsis.org](http://www.arabidopsis.org). The protein function column displays the presumed biological function evidenced by the associated publications. Where possible, the reference describing the cloning and the latest related publication is given.

AGI	Gene name	Protein function	References
AT4G16280	<i>FCA</i>	RNA-binding	Macknight <i>et al.</i> , 1997
AT5G13480	<i>FY</i>	Polyadenylation factor	Simpson <i>et al.</i> , 2003
AT3G10390	<i>FLOWERING LOCUS D (FLD)</i>	HDAC-associated protein	Sanda and Amasino, 1996a
AT4G02560	<i>LUMINIDEPENDENS (LD)</i>	Homeodomain protein	Lee <i>et al.</i> , 1994a, b
AT2G43410	<i>FPA</i>	RNA-binding	Schomburg <i>et al.</i> , 2001
AT2G19520	<i>FVE</i>	MSI homologue	Kim <i>et al.</i> , 2004
AT3G04610	<i>FLOWERING LATE KH MOTIF (FLK)</i>	RNA-binding	Lim <i>et al.</i> , 2004

*FVE* encodes an MSI1 homologue (Ausin *et al.*, 2004; Kim *et al.*, 2004) and *FLD* a putative homologue of the lysine-specific histone demethylase LSD1 (Sanda and Amasino, 1996a; He *et al.*, 2003; Shi *et al.*, 2004, 2005; Metzger *et al.*, 2005). Both *FVE* and *FLD* have been implicated in histone deacetylation complexes (He *et al.*, 2003; Ausin *et al.*, 2004). *FCA*, *FPA* and *FLK* all contain putative RNA-binding proteins; the otherwise unrelated *FCA* and *FPA* proteins contain RRM-domains (Macknight *et al.*, 1997; Schomburg *et al.*, 2001), while *FLK* contains three KH domains (Lim *et al.*, 2004; Mockler *et al.*, 2004). *FY* is homologous to Pfs2p, an essential polyadenylation and 3'-end processing factor from yeast (Simpson *et al.*, 2003). *LD* is a homeodomain protein with unknown function (Lee *et al.*, 1994a). Genetic and biochemical analysis indicates that *FCA*, *FY* form one sub-pathway, while *FPA* and *FVE* might form another (Koornneef *et al.*, 1998; Simpson *et al.*, 2003). Since the *ld* and *fld* mutations are strongly suppressed by *Ler-FLC* the interaction of these mutations with other autonomous promotion pathway mutations has not been reported so far. Thus the autonomous promotion pathway consists of a diverse set of biochemical functions, some of which play generic roles in other organisms.

### FCA interaction with FY mediates changes in FCA transcript processing

*FCA* physically interacts with *FY* through a WW domain in the C-terminal part of *FCA* and PPLPP residues in the C-terminal part of *FY* (Simpson *et al.*, 2003) (Fig. 2A, B). The C-terminal part of *FY* is an additional domain of the protein not present in the yeast Pfs2p and so may have evolved to play a regulatory role. The *FY* homologues in humans and mouse also have an unrelated 3' extension relative to the yeast protein so this could be quite a general phenomenon. Functional evidence to show that the homology of *FY* to yeast poly A/3' processing factor is meaningful and was found through analysis of *FCA* regulation. Four *FCA* transcripts are present in cells, in one of which polyadenylation occurs within the third intron (Fig. 2C).



**Fig. 2.** Domain structure of *FCA* and *FY* proteins and autoregulation of *FCA* transcription. (A) Domain structure of *FCA*. *FCA* contains two N-terminal RRM domains and a C-terminal WW (typified by two conserved tryptophan residues) domain. (B) *FY* domain structure consisting of seven N-terminal WD-40 motifs and two C-terminal PPLPP (proline and leucine residues) repeats. (C) A northern blot analysis showing different *FCA* transcripts. The abundance of the  $\alpha$ ,  $\beta$ , and  $\gamma$  transcripts are compared in wild-type (*Ler*) and *fca-1* and *fy-1* mutants in wild-type or *35S::FCA $\gamma$*  over-expressing background. The membrane was stripped and re-probed with  $\beta$ -*TUBULIN* as a loading control.

Over-expression of *FCA* promotes the use of this proximal polyadenylation site so reducing the abundance of the full-length *FCA* transcript (*FCA $\gamma$* ), the only transcript that produces a functional *FCA* protein. The *FCA*/*FY* interaction is required for the use of this proximal poly A site and so for the negative autoregulation of *FCA*. Thus, this negative feedback is missing in *fy-1* (Macknight *et al.*, 2002; Quesada *et al.*, 2003), Fig. 2C. The phenotype of

*fy-1*, the only *fy* allele available for many years, is a slight delay in flowering. When a second allele was identified from the Salk T-DNA collection it was also found to be late-flowering with increased *FLC* levels. However, both these alleles were still found to express the N-terminal part of the FY protein containing the seven WD repeats, which constitutes the whole protein in yeast (Henderson *et al.*, 2005). To assess the phenotype of a true *fy* null mutant, alleles from the NSF TILLING population (McCallum *et al.*, 2000) were characterized. *fy-4* was found to carry a premature stop codon at the end of the first WD repeat and this prevented any FY protein being made. *fy-4* mutants are embryo lethal suggesting that, as in yeast, FY has a general role in *Arabidopsis* and FCA might recruit specific transcripts to the canonical polyadenylation machinery (Henderson *et al.*, 2005).

Pleiotropic functions for FY in development had been previously suggested by the genetic analysis of the autonomous promotion pathway mutants (Koornneef *et al.*, 1998). An *fy-1*, *fpa* double mutant had never been recovered, suggesting that it may cause embryo lethality. By contrast, *fca fy-1* and *fca fpa* double mutants are viable. Analysis of plants heterozygous for *fy-1* but homozygous for *fpa* led to a high incidence of aborted seed and when these plants were crossed reciprocally with *Ler* it became clear that both *fy* and *fpa* cause weak gametophytic defects which, when combined together, cause synergistic lethality (Henderson *et al.*, 2005).

A likely model for how FCA and FY regulate *FLC* is through a similar mode of action to that by which they regulate FCA. However, only one *FLC* transcript accumulates to significant levels in *Arabidopsis* seedlings. So if FCA/FY promote polyadenylation at a proximal site the resultant transcript is rapidly degraded. The mode of action of FCA/FY on *FLC* is being investigated using a suppressor mutagenesis approach where the *FCA* cDNA has been over-expressed from a 35S promoter. The resultant early-flowering line was mutagenized and late-flowering individuals identified (V Quesada, F Liu, C Dean, unpublished data). Analysis of the genes identified, together with molecular analysis of low abundance *FLC* transcripts, should reveal the molecular mechanism involved.

### Additional roles for the autonomous promotion pathway

The current view of the autonomous promotion pathway is a series of semi-redundant sub-pathways which, based on the embryo lethality phenotype of null *fy* mutants and the observation that mutations in *FVE* have also been identified in screens for cold stress signalling (Kim *et al.*, 2004), are unlikely to be floral specific. In order to define the activities of this pathway further, a microarray analysis was undertaken comparing wild-type and *fca* mutant seedling gene

expression. A previous microarray analysis had used a custom array of 13 000 genes (Wilson *et al.*, 2005). This analysis examined all the mutants of the autonomous pathway plus members of the vernalization and photoperiod pathways. The overall conclusion was that there were very few changes in gene expression in the mutants and the genes that did change were involved in defence and metabolism. It was decided to repeat some of the array experiments using the commercially available and widely used Affymetrix Arabidopsis 8k microarrays. These provide an excellent opportunity to examine a large fraction of the *Arabidopsis* transcriptome simultaneously and compare experiments between many laboratories (Schmid *et al.*, 2005; Zimmerman *et al.*, 2005). However, data created in microarray experiments are susceptible to influences from plant growth conditions, RNA handling, and hybridization. A commonly implemented method to ensure generation of reliable data is to use duplicates or triplicates of the same but independently obtained samples. To test which of the observed changes are shared by the replicates, the data can be subjected to a principal component analysis or a condition tree analysis, both features in Genespring software. The results were compared from eight arrays: duplicates of Columbia, *fca-9* (a Col *fca* allele), *Ler*, and *fca-1* (the strongest *Ler* allele). Using the tests to study the comparability of the microarrays analysed, a batch effect became obvious. Instead of grouping as expected according to their genotypes, the similarity of the datasets was mostly determined by the experiment date. This led to the formation of four major correlation groups (CGs) in the condition tree analysis. The observation that there was a wild-type control and an *fca* mutant microarray within each of the CGs gave the potential to improve the data and circumvent the batch effect problem. It was reasoned that if the batch effect superimposes the expression differences caused by the genotype and leads to the observed grouping by date, it might be possible to extract the interesting, overlaid genotypic differences using a three step mechanism. First, the comparison between *fca* and wild-type within each separate CG was regarded as a way to subtract a vast amount of the superimposed changes and to deliver the first two subsets of 1.5-fold up- and down-regulated gene lists. This was followed with the identification of those genes which were shared within the two 1.5-fold up- and down-regulated gene lists obtained for each of the two ecotypes examined (CG 1 and 3 are in *Ler*, CG 2 and 4 are in Col-0). This produced four gene-lists; 1.5-fold up- and down-regulated for each ecotype, comprising each between 400 and 600 genes. Since an *fca* mutation shows a similar phenotype in both Col-0 and *Ler*, it was reasoned that it would be appropriate to assume similar transcript changes across the accessions. Therefore it was decided to check the gene-lists obtained in step two for shared up- and down-regulated genes across the accessions as the third step. In theory, the analysis of accession-independent

transcriptome changes is a strength of the research because a more general regulatory network can be identified. Confidence was gained in the approach because two genes known to be affected by *fca*, *FLC* and *SOC1*, were consistently found in their respective gene lists after each step. Our final gene lists comprised 31 up- and 37 down-regulated genes in *fca* mutant versus wild-type plants (Tables 2, 3).

The expression profile of these genes was validated by RNA gel blots. Primers were designed against the known Col-0 cDNA sequence to obtain specific DNA probes for 21 of the 68 differentially regulated genes. Twelve were successfully analysed and despite the strategy aimed at identifying genes mis-expressed in both *Ler* and Col-0 the only changes in gene expression that could be confirmed in both *Ler* and Col-0 were *FLC* and *SOC1*. The observed pattern of bands obtained with the *SOC1* specific probe inversely correlates with the *FLC* abundance and is highest in *flc* mutants and in the *35S::FCA $\gamma$*  overexpressing line (Fig. 3).

Several other changes in gene expression could be confirmed, but only in an accession-specific manner. The genes encoding subtilase, PPR, DNA repair, and the RING-finger protein (Table 2) showed the expected RNA abundance patterns in Col-0 and *fca-9* (Fig. 2), whereas changed *SAC1* expression was confirmed in a *Ler/fca-1* comparison (Table 3, data not shown). For the other probes analysed, *RAD50*, *expressed Protein* (At4g19970), *PAP1*, *similar to FAR1*, and *ATRPAC14* (Tables 2, 3) the pattern of expression predicted from the arrays was not verified (data not shown). The observed accession-specific differences were unexpected given the analysis designed to identify regulatory patterns shared by both accessions. It is possible that the observed batch effect was too large to be overcome with the methods used or that differences in the genomic sequence between the two accessions interfered with the comparison both during hybridization of the microarray and validation on the northern blots.

The aim of the microarray experiments was to identify FCA targets that are required for *FLC* regulation or are direct targets independent of *FLC*. Therefore the expression of the Col-0 verified genes was compared in *fca-9* and *fca-9 flc-3*. If the expression was the same in both, it was reasoned that the gene was a candidate we were interested in and was not dependent on *FLC* function. The genes encoding subtilase, PPR, DNA repair, and RING-finger proteins were equally down-regulated in *fca-9* and the *fca-9 flc-3* and this misregulation was not observed in *flc-2* (Fig. 3). These genes may function downstream of FCA, but upstream of *FLC* or they may be FCA targets that do not play a role in flowering time regulation. Analysis of the phenotypes of knock-outs of these genes will help elucidate this. The four genes were down-regulated in the *fca* mutant suggesting that, unlike the situation for *FLC*, FCA may promote their expression. However, it was not known if these effects are direct.

**Table 2.** Down-regulated genes in *fca* versus wild-type plants

The genes are listed with those showing the greatest fold change at the bottom of the table. The FOLD column displays the fold-change in *fca* mutants versus wild-type plants. The AGI column displays the associated gene identifier obtained after blasting the sequence of the Affymetrix probe set, obtained from www.affymetrix.com, against the *Arabidopsis* cDNA and UTR database at www.arabidopsis.org. The Function column provides some information about the misregulated genes. The information was found in a database search at www.arabidopsis.org for each individual gene. Where possible, an annotated gene name is given in this column. For genes highlighted in grey northern blot results are shown (Fig. 3).

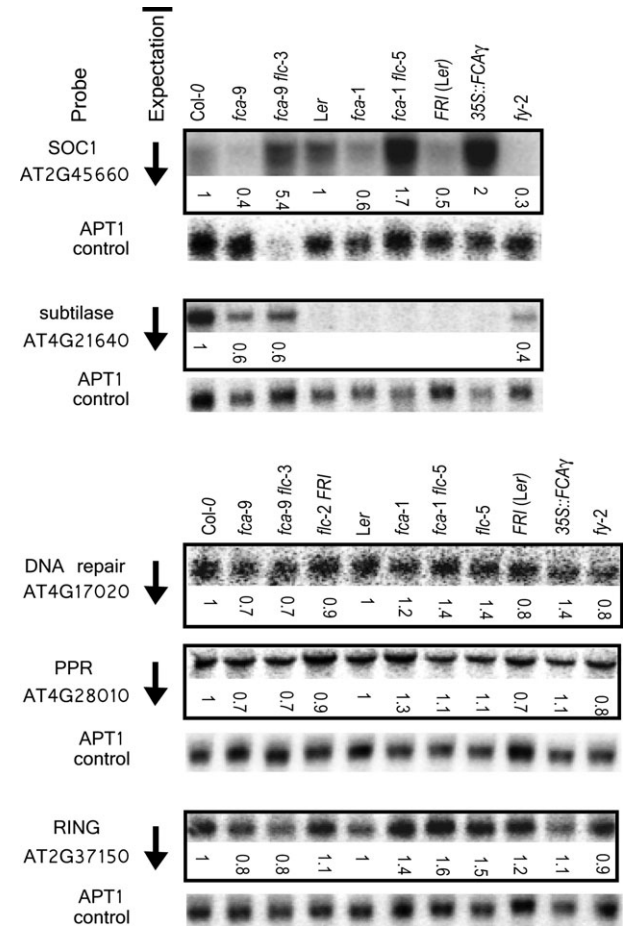
FOLD	AGI	Function
1.8	AT2G02710	PAC motif containing protein
2.1	AT2G37260	<i>TRANSPARENT TESTA GLABRA2</i> ( <i>TTG2</i> )
2.2	AT2G27650	Ubiquitin carboxyl-terminal hydrolase related protein
2.3	AT2G37150	Zinc finger, C3HC4-type RING finger protein
2.3	AT2G43620	Chitinase
2.4	AT5G27110	PPR-repeat containing protein
2.4	AT4G37370	Member of CYP81D
2.4	AT4G21340	Similar to ethylene-responsive proteins
2.4	AT4G14990	Expressed protein
2.5	AT4G17020	DNA repair, transcription factor related
2.5	AT4G32920	Glycine-rich protein
2.5	AT2G23070	Similar to casein kinase II
2.6	AT2G22050	Kelch repeat-containing F-box protein
2.8	AT2G29540	DNA-directed RNA polymerase I(A) and III(C) 14 kDa subunit ( <i>ATRPAC14</i> )
3.1	AT4G11020	Expressed protein
3.2	AT2G39410	Hydrolase
3.3	AT2G41210	Similar to phosphatidylinositol-4-phosphate 5-kinase proteins
3.4	AT4G00320	F-box family protein
3.5	AT1G67260	<i>TCP DOMAIN CONTAINING1</i> ( <i>TCPI</i> )
3.6	AT1G61960	Mitochondrial transcription termination factor-related
3.7	AT1G10330	PPR-repeat containing protein
3.9	AT2G27520	F-box family protein
4.0	AT4G18960	<i>AGAMOUS</i> ( <i>AG</i> )
4.0	AT1G30740	FAD-binding domain containing protein
4.1	AT2G45660	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1</i> ( <i>SOC1</i> )
4.1	AT2G14580	<i>PATHOGEN RELATED BASIC1</i> ( <i>PRB1</i> )
4.1	AT4G21640	Subtilase family protein
4.2	AT5G14750	<i>WEREWOLF</i> ( <i>WER</i> )
4.2	AT4G22650	Hypothetical protein
4.3	AT2G38185	Zinc finger, C3HC4-type RING finger
4.4	AT2G20815	Expressed protein
4.4	AT4G22640	Expressed protein
5.2	AT4G26560	<i>CALCINEURIN B LIKE7</i> ( <i>CBL7</i> )
5.2	AT1G23510	Expressed protein
5.8	AT4G29340	<i>PROFILIN4</i> ( <i>PRF4</i> )
6.6	AT4G19990	Similar to FAR RED IMPAIRED1 ( <i>FAR1</i> )
6.7	AT4G28010	PPR-repeat containing protein

**Table 3.** *Up-regulated genes in fca versus wild-type plants*

The genes are listed with those showing the greatest fold change at the bottom of the table. The FOLD column displays the fold-change in *fca* mutants versus wild-type plants. The AGI column displays the associated gene identifier obtained after blasting the sequence of the Affymetrix probe set, obtained from [www.affymetrix.com](http://www.affymetrix.com), against the *Arabidopsis* cDNA and UTR database at [www.arabidopsis.org](http://www.arabidopsis.org). The Function column provides some information about the misregulated genes. The information was found in a database search at [www.arabidopsis.org](http://www.arabidopsis.org) for each individual gene. Where possible, an annotated gene name is given in this column.

FOLD	AGI	Function
1.7	AT4G08390	L-ascorbate peroxidase, stromal
2.1	AT4G02420	Lectin protein kinase
2.2	AT4G35600	<i>CONNEXIN 32</i>
2.3	AT1G09090	Respiratory burst oxidase protein
2.3	AT2G06220	Pseudogene
2.4	AT1G56650	<i>PRODUCTION OF ANTHOCYAN PIGMENT1 (PAP1)</i>
2.5	AT4G30860	SET domain-containing protein
2.6	AT4G09790	F-box family protein
2.7	AT1G04370	Encodes a member of the ERF family proteins
2.7	AT1G04580	<i>ARABIDOPSIS ALDEHYDE OXIDASE4 (AAO4)</i>
2.8	AT1G16300	<i>GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE</i>
2.8	AT1G22620	<i>SAC DOMAIN PHOSPHOINOSITE PHOSPHATASE (SAC1)</i>
2.9	AT2G47780	Rubber elongation factor protein related
3.0	AT2G42730	F-box family protein
3.0	AT4G23130	<i>RECEPTOR LIKE PROTEIN KINASE 6 (RLK6)</i>
3.1	AT4G00540	<i>PUTATIVE C-MYB TRANSCRIPTION FACTOR (PC-MYB2)</i>
3.1	AT2G23050	Phototropic-responsive NPH3 family protein
3.2	ATMG00660	Hypothetical protein
3.4	AT1G49570	<i>ARABIDOPSIS THALIANA PEROXIDASE 5a (ATP5)</i>
3.5	AT2G31970	<i>RAD50</i>
3.5	AT4G01930	<i>BASIC PENTACYSSTEINE1 (BPC1)</i>
3.8	AT4G33020	<i>ZINC IRON PERMEASE 9 (ZIP9)</i>
3.9	AT2G20650	Zinc finger, C3HC4-type RING finger
4.2	AT2G12390	CACTA-like transposase family
4.2	AT4G23540	Expressed protein
4.3	AT2G05910	Expressed protein
4.3	AT1G60530	Dynamain family protein
4.4	AT1G60950	<i>FERREDOXIN A (FED A)</i>
4.5	AT2G22030	Kelch repeat containing F-box family protein
4.6	AT4G19970	Expressed protein
5.0	AT2G07030	Mutator like transposase family
24.9	AT5G10140	<i>FLOWERING LOCUS C (FLC)</i>

Transcript levels of the four validated genes were examined in a Col-0 *fy* mutant background (*fy-2*) (Fig. 3). The down-regulation in *fy-2* of all four FCA targets is consistent with FCA and FY functioning together *in vivo* to regulate these transcripts, but again, these effects may not be direct. Taken together, these findings point towards a model in which FCA and FY share multiple targets *in vivo* and play a more general role in gene expression than just regulating *FLC*.



**Fig. 3.** Northern blot analysis in order to validate the expression of candidate genes derived from Table 2. The results of specific probes against five candidate genes are shown. They were used to probe four different membranes loaded with either nine or 11 genotypes. The expected direction of change in expression in *fca* mutants is marked by an arrow. Membranes were stripped and reprobed with *ADENOPHOSPHORIBOSYL TRANSFERASE 1 (APT1)* as a loading control. The quantified signal intensity of the *APT1* control was used to normalize the quantified probe specific signals and they are expressed relative to their respective wild-type control, which was given the value 1 (Col-0 for *fca-9*, *fca-9 fca-3*, *flc-2 FRI*, and *fy-2* but *Ler* for *fca-1*, *fca-1 fca-5*, *flc-5*, *FRI*, and *35S::FCA $\gamma$* ).

## Conclusion

Mutants of *Arabidopsis thaliana* that flower late in long and short day photoperiods and whose phenotype can be corrected by vernalization, have led to the identification of genes functioning in the autonomous promotion pathway. The pathway functions to repress expression of the floral repressor *FLC*. However, during the cloning and biochemical analysis of some of the autonomous promotion pathway components a more general cellular role for some of the components has emerged. It is possible that, since *FLC* acts as a quantitative repressor, any changes in its expression would have phenotypic consequences, so general regulators would be identified as specific flowering time regulators. The microarray analysis described here

has provided insight into the additional targets of the autonomous promotion pathway. Their analysis will further define the functional relationship of some of the autonomous promotion pathway mutants and continue to test the notion that the pathway has more general functions than flowering time control alone.

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