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Abstract

EARLI1 encodes a 14.7 kDa protein in the cell wall, is a member of the PRP (proline-rich protein) family and has multiple functions, including resistance to low temperature and fungal infection. RNA gel blot analyses in the present work indicated that expression of EARLI1-like genes, *EARLI1*, *At4G12470* and *At4G12490*, was down-regulated in Col-*FRI*-Sf2 RNAi plants derived from transformation with *Agrobacterium* strain ABI, which contains a construct encoding a double-strand RNA targeting 8CM of *EARLI1*. Phenotype analyses revealed that Col-*FRI*-Sf2 RNAi plants of *EARLI1* flowered earlier than Col-*FRI*-Sf2 wild-type plants. The average bolting time of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants was 39.7 and 19.4 days, respectively, under a long-day photoperiod. In addition, there were significant differences in main stem length, internode number and rosette leaf number between Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants. RT-PCR showed that EARLI1-like genes might delay flowering time through the autonomous and long-day photoperiod pathways by maintaining the abundance of *FLC* transcripts. In Col-*FRI*-Sf2 RNAi plants, transcription of *FLC* was repressed, while expression of *SOC1* and *FT* was activated. Microscopy observations showed that EARLI1-like genes were also associated with morphogenesis of leaf cells in *Arabidopsis*. Using histochemical staining, EARLI1-like genes were found to be involved in regulation of lignin synthesis in inflorescence stems, and Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants had 9.67% and 8.76% dry weight lignin, respectively. Expression analysis revealed that cinnamoyl-CoA reductase, a key enzyme in lignin synthesis, was influenced by EARLI1-like genes. These data all suggest that EARLI1-like genes could control the flowering process and lignin synthesis in *Arabidopsis*.

Introduction

More than 500 proteins from different plant species have been classified into the 8CM superfamily, having eight cysteine residues in their homologous region. *Arabidopsis* encodes 105 8CM proteins, 23 of these that are similar to EARLI1 in structure were placed in the HyPRP (hybrid proline-rich protein) family because they contain a proline-rich domain (PRD) before the 8CM motif. The HyPRP family plays an important role in diverse plant defence responses (**Josè-Estanyol *et al.* 2004**).

The name *EARLY ARABIDOPSIS ALUMINIUM-INDUCED GENE1* (*EARLI1*) is because it can be induced by aluminium (**Richards *et al.* 1998**). *EARLI1* encodes a 14.7 kDa protein containing a hydrophilic N-terminal PRD with high similarity to HyPRPs and extensin in the cell wall, and a hydrophobic C-terminal conservative 8CM that might have functional connections with the plasma membrane (**Zhang & Schläppi 2007**). *At4G12470*, *At4G12490* and *EARLI1* are closely related paralogues, clustered in a tandem array at the same chromosomal locus and referred to as EARLI1-like genes. The coding products of these genes possess 50–65% identity with DEA1 of tomato; all of them contain signal peptide sequences and are also classified into the lipid transfer protein (LTP) family (**Weyman *et al.* 2006**).

Previous research indicated that *EARLI1* could also be induced by low temperature. Its expression could be activated by long or short cold treatment, and the higher transcription level could be maintained more than 20 days when plants were transferred to room temperature (**Wilkosz & Schläppi 2000**). Electrolyte leakage assays showed that transgenic *Arabidopsis* over-expressing *EARLI1* leaked fewer electrolytes than wild-type plants under freezing damage conditions, suggesting that *EARLI1* was related to stability between the plasma membrane and cell wall (**Bubier & Schläppi 2004**). Similar results were obtained in yeast freezing experiments. In comparison to yeast cells transformed with an empty vector, yeast cells harbouring EARLI1-like genes had higher survival rates. Immunofluorescence observation of EARLI1-GFP in transgenic *Arabidopsis* showed that EARLI1 was localised on the cell wall, and Western blot analyses further confirmed the involvement of EARLI1 in maintenance of the stability between plasma membrane and cell wall through binding with other proteins or forming complexes (**Zhang & Schläppi 2007**). Plants suffering low temperature stress can accumulate abscisic acid (ABA), however, *EARLI1* is insensitive to ABA and it expression cannot be induced by exogenous ABA treatment (**Bubier & Schläppi 2004**).

Increasing evidence suggests that EARLI1-like genes might have multiple functions in the life cycle of *Arabidopsis*; one of which is resistance to pathogens. **Eulgem *et al.* (2004)** analysed the expression status of 8000 *Arabidopsis* genes after infection with *Peronospora parasitica*, and found transcription of a large number of genes, including *EARLI1*, was enhanced transiently or continuously. Simulation experiments of *Botrytis cinerea* infection with cutinase indicated that transgenic *Arabidopsis* over-expressing EARLI1-like genes had resistance to this fungus pathogen, and microarray data showed that *At4G12470* might play the most important role in this process, suggesting that *At4G12470* was mainly involved in regulation of pathogen defence. In addition, EARLI1-like genes may possess mutual redundancy functions (**Chassot *et al.* 2007**).

Over-expression of AtNHX1, the vacuolar Na^+/H^+ antiporter of *Arabidopsis*, could significantly increase tolerance to salt stress. In the *nhx1* mutant, expression of *At4G12470* and *EARLI1* somewhat decreased, indicating that they probably participate in plant response to salt stress in cooperation with *AtNHX1* (**Sottosanto *et al.* 2004, 2007**). Abscisic acid-responsive protein ABR17 of *Pisum sativum* is a member of the pathogenesis-related protein PR10 family that responds to multiple biotic and abiotic stresses. Damage caused by 100 mm NaCl treatment to transgenic *Arabidopsis* over-expressing *ABR17* was fairly mild compared to that in wild-type plants, but expression of EARLI1-like genes was enhanced in the transgenic plants, further proving that EARLI1-like genes are related to a regulatory mechanism of plant response to salt stress (**Krishnaswamy *et al.* 2008**). These results

suggest that as proteins located on the cell wall, the first defensive barrier of the plant, the coding products of EARLI1-like genes probably have diverse and potential functions in resistance to biotic and abiotic stresses, and might be involved in every stage of *Arabidopsis* development and growth. In the present work, RNA interference (RNAi) lines were used to analyse the influence of EARLI1-like genes on flowering time and lignin systhesis of *Arabidopsis*. We found that the down-regulation of EARLI1-like genes led to a decrease in lignin content and earlier flowering.

Materials and Methods

Plant material

Late-flowering *Arabidopsis* ecotype Col-*FRI*-Sf2 containing the dominant San Feliu (Sf2) allele of *FRIGIDA* (*FRI*) and a dominant Columbia (Col) allele of *FLOWERING LOCUS C* (*FLC*), and three independent homozygous RNAi lines of *EARLI1* in the same genetic background were used as experimental material. Construction of RNAi lines was described previously (**Zhang & Schläppi 2007**). After sterilisation with 70% ethanol and 0.1% $HgCl_2$, seeds were incubated at 4 °C for 2–4 days to break dormancy and were germinated on agar-solidified half-strength MS medium with or without 50 $mg \cdot l^{-1}$ kanamycin. The germination was counted after 7 days and growth was compared at different stages.

RNA gel blot and RT-PCR analysis of gene expression

Total RNA from 0.2 g of Col-*FRI*-Sf2, and its *EARLI1* RNAi seedlings, grown for 4 weeks in a long-day photoperiod (16-h light/8-h dark cycles at 22 °C day/20 °C night), was extracted with the E.Z.N.A. total RNA kit II (Omega, Norcross, GA, USA). RNA was quantified with ultraviolet spectrophotometry and separated in 1.2% formaldehyde/3-(*N*-morpholino) propanesulphonic acid gel containing ethidium bromide. Genomic DNA was extracted according to the instructions of the Universal Genomic DNA extraction kit Ver.3.0 (TaKaRa, Dalian, China). For probe preparation, genomic DNA was used as template in amplification of *EARLI1*, *At4G12470* and *At4G12490* fragments because no intron could be found in these genes. PCR primers for *EARLI1* were 5′-TTTCTTCGCCCTTAACATCA-3′ and 5′-AAGCCAGACGGAACCTTTC-3′; PCR primers for *At4G12470* were 5′-GCTTCAAAGAACTCAGCCTC-3′ and 5′-TACAAAGCTTGGAAGGCG-3′. The probe for *At4G12490* was prepared by PCR with the primer pair 5′-TCTTTGCCCTCAACATCCT-3′ and 5′-GCACATTCGCACATACACC-3′. DIG High Prime DNA Labelling and Detection Starter kit I was used for probe labelling and RNA gel blotting (Roche, Penzberg, Germany). The substrates of alkaline phosphatase, NBT/BCIP, were used in signal dectection. In RT-PCR analysis of *CCR1* expression, total RNA was extracted from inflorescence stems of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants according to instructions for the Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesised with the RevertAid First Strand cDNA Synthesis kit #1621 (Fermentas, Burlington, ON, Canada). RT-PCR primers for *CCR1* were 5′-TCCTCCATTGGTGCCGTCTA-3′ and 5′-AGGGTTCTTCTCGTCCTTGC-3′. RT-PCR primers for *ACT8* were 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-TCCGAGTTTGAAG AGGCTAC-3′. In RT-PCR analysis of flowering-related genes, total RNA was extracted from 2-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants grown in a long-day photoperiod. RT-PCR primers for *FLC* were 5′-GCATCCGTCGCTCTTCTCGT-3′ and 5′-AGGGCAGTCTCAAGGTGTTCC-3′; for *FRI* were 5′-TGTAGCAAAGGTCTGCGTAA-3′ and 5′-CACCTTCCCTTTACCACGAT-3′; for *SOC1* were 5′-CCTTTGAGCTCTCAGTGCTT-3′ and 5′-CTTGGGCTACTCTCTTCATC-3′; and for *FT* were 5′-GTTGTTGGAGACGTTCTTG-3′ and 5′-CCTCCGCAGCCACTCTCCC-3′.

Influence of *EARLI1* on cell structure and lignin synthesis of *Arabidopsis*

Roots and cotyledons of 1-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi seedlings were used as material in TEM (transmission electron microscope) observations. Leaves of 4-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants were used as material in SEM (scanning electron microscope) observations. In other cytological experiments, inflorescence stems and roots of 8-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants were used as material. One-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi seedlings were fixed in 2% glutaraldehyde for 4 h and rinsed with 0.2 m phosphate buffer (pH 7.4) three times at 10-min intervals. The seedlings were post-fixed in 1% OsO_4 at 4 °C for 1–2 h. After rinsing three times with 0.2 m phosphate buffer (pH 7.4), the material was dehydrated in a graded acetone series and embedded in Epon812 (Serva, Heidelberg, Germany). Sections of 60–80 nm were stained with lead citrate and uranyl acetate and observed under a JEOL JEM-2000EX TEM. Samples used in SEM were fixed in FAA, rinsed with water, dehydrated in an alcohol series, infiltrated with ethanol:isoamyl acetate, critical-point dried in CO_2, mounted on aluminium stubs, sputter-coated with gold and checked with a Hitachi S2570 SEM. In preparation of freehand sections, inflorescence stems of 8-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants were transected into segments of 3–5 cm and fixed for 1 h with FAA fixative containing 3.7% formaldehyde (v/v), 5% acetic acid (v/v) and 50% ethanol. The samples were placed on to a clean glass slide and cut into sections of 0.1–0.2 mm, then stained with 1% phloroglucinol for 5 min. One drop of 75% HCl was added subsequently and the sections were examined under a light microscope. Semi-thin root tip sections of 1–2-µm thick from 8-week-old plants were stained with 1% toluidine blue. In determination of lignin content, inflorescence stems of 8-week-old *Arabidopsis* were homogenised in liquid N_2 and extracted with 70% methanol to colourless. An aliquot of 0.2 g of dried insoluble substances was hydrolysed with 4 ml 72% sulphuric acid for 4 h and diluted with 112 ml distilled water. After autoclaving at 121 °C for 1 h, the samples were filtered in a sand core crucible (W1) and dried to constant weight (W2), so that lignin content = (W2 − W1) × 100%/0.2. The data from three independent RNAi lines were statistically analysed with Student's *t*-test.

Statistical analyses of indices related to flowering time

Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants grown on half-strength MS medium in long-day conditions were used as material in statistical analyses of indices related to flowering time. As a flowering plant, *Arabidopsis* produces inflorescences after the end of the vegetative growth and the beginning of reproductive growth, and flowers in the subsequent period. Because vegetative growth is accompanied with a continuous increase in number and size of organs such as roots and leaves, flowering time is usually estimated by counting the number of rosette leaves when the floral transition is evident (visible appearance of floral buds) or when bolting is initiated, while the length of the inflorescence stem, internode number, lateral stem number and cauline leaf number can be used as indices to judge the timing of reproductive growth. In this work, the length of the main stem, number of internodes, number of lateral stems, number of rosette leaves and number of cauline leaves of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants grown in long-day conditions were counted. The data from three independent RNAi lines were statistically analysed with Student's *t*-test.

Results

Down-regulation of *EARLI1*, *At4G12470* and *At4G12490* expression by RNA interference

RNA interference is an important method in identification of gene function, and can be used to effectively inhibit expression of target genes. In the present work, total RNA was extracted from 4-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants. Northern blotting analyses showed *EARLI1* expression was repressed significantly by RNA interference in comparison to wild-type plants under long-day conditions, and almost no *EARLI1* mRNA could be detected (**Fig. 1**). Because the RNAi construct was designed based on the conservative 8CM domain, expression of EARLI1-like genes should also be down-regulated. Expression analyses of *At4G12470* and *At4G12490* confirmed this, but the inhibition degree of RNA interference to *At4G12470* and *At4G12490* was lower than that to *EARLI1*. *At4G12470* and *At4G12490* still expressed in RNAi lines to some extent (**Fig. 1**). All these data are consistent with the results of **Zhang & Schläppi (2007)**. It should be noted that *EARLI1* could not express in short-day photoperiods.

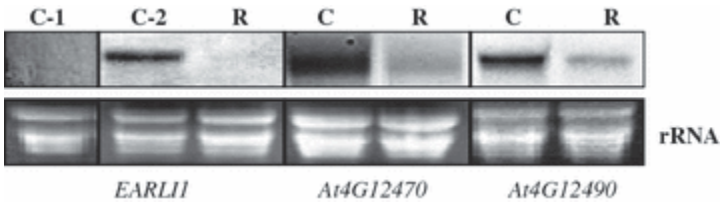

Figure 1 Expression of *EARLI1*, *At4G12470* and *At4G12490* in 4-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants. C, Col-*FRI*-Sf2; R, Col-*FRI*-Sf2 RNAi; 1, 2 represent short-day and long-day photoperiods, respectively.

Influence of *EARLI1* on cell structure and lignin synthesis of *Arabidopsis*

With the EARLI1-GFP fusion construct, **Zhang & Schläppi (2007)** found that EARLI1 was located on the cell wall. In order to determine the relationship of EARLI1 with morphogenesis of *Arabidopsis* cells, microscopic structures of roots and leaves of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi seedlings were observed with TEM. The average cell wall thickness of cotyledon epidermal cells in 1-week-old Col-*FRI*-Sf2 seedlings was 150 nm, while the average cell wall thickness of cotyledon epidermal cells in 1-week-old Col-*FRI*-Sf2 RNAi seedlings was 300 nm, but the staining intensity of the cuticules of cotyledon epidermal cells in Col-*FRI*-Sf2 RNAi seedlings was weak in comparison with that of Col-*FRI*-Sf2 seedlings, indicating that EARLI1-like genes were related to cutin synthesis (**Fig. 2A and B**). Moreover, the average diameter of tracheary elements in the root vascular bundle of Col-*FRI*-Sf2 RNAi plants was reduced to 4.25 μm in comparison with 6.78 μm in wild-type plants, and there were many inclusions in chloroplasts of leaf vein cells in Col-*FRI*-Sf2 RNAi plants (**Fig. 2C–E**). SEM observations of leaves from 4-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants showed that the shape of leaf cells of Col-*FRI*-Sf2 RNAi plants was stretched and tenuous in comparison with wild-type cells (**Fig. 3B**), coinciding with the shape of leaves at this stage (**Fig. 3E**). The trichome base of the Col-*FRI*-Sf2 leaf was round, while the base of some trichomes in Col-*FRI*-Sf2 RNAi plants was serrate, suggesting that EARLI1-like genes were possibly involved in development of trichomes (**Fig. 3C and D**). Some RNAi plants was comparatively small and weak, had no main inflorescence stem, and their siliques were curved (**Fig. 3F and G**).

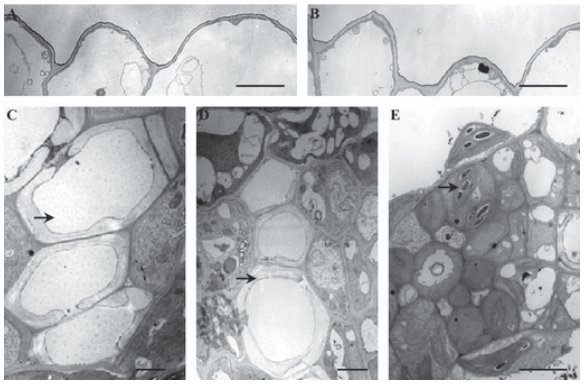

Figure 2 Microscopy observations of root and cotyledon cells in 1-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants. A, Epidermal cells of Col-*FRI*-Sf2 cotyledon, bar = 2 μm; B, Epidermal cells of Col-*FRI*-Sf2 RNAi cotyledon, bar = 2 μm; C, Tracheary elements in root vascular bundle of Col-*FRI*-Sf2 plant, bar = 2 μm; D, Tracheary elements in root vascular bundle of Col-*FRI*-Sf2 RNAi plant, bar = 2 μm; E, Leaf vein cells in Col-*FRI*-Sf2 RNAi plant, bar = 2 μm.

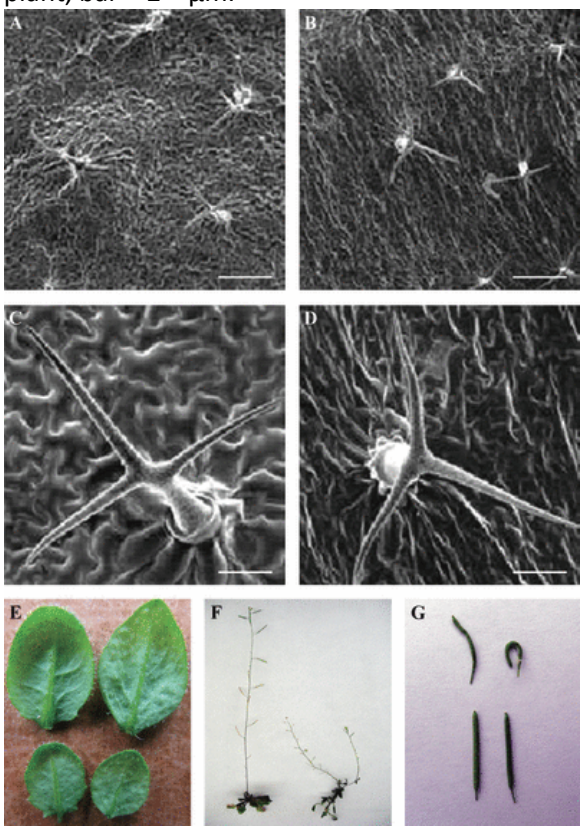

Figure 3 SEM and phenotype observations of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants. A, Leaf epidermis of 4-week-old Col-*FRI*-Sf2 plants, bar = 220 μm; B, Leaf epidermis of 4-week-old Col-*FRI*-Sf2 RNAi plants, bar = 220 μm; C, Trichomes in 4-week-old Col-*FRI*-Sf2 leaf, bar = 52 μm; D, Trichomes in 4-week-old Col-*FRI*-Sf2 RNAi leaf, bar = 52 μm; E, leaf shape, the left lane is leaves from Col-*FRI*-Sf2, the right lane is leaves from Col-*FRI*-Sf2 RNAi; F, Plant shape, left is Col-*FRI*-Sf2, right is Col-*FRI*-Sf2 RNAi plant; G, silique shape, upper is Col-*FRI*-Sf2 RNAi, under is Col-*FRI*-Sf2.

Because EARLI1 was localised to the cell wall, whether it functioned in modification of the structure of cell wall by changing the composition was analysed histochemically. The inflorescence stems of 8-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants were stained with phloroglucinol. The lignified cell wall of interfascicular fibre cells in Col-*FRI*-Sf2 inflorescence stems was thicker than that in RNAi lines

(**Fig. 4A**), and the cell wall of interfascicular fibre cells in some RNAi plants was orange rather than red (**Fig. 4B and C**). This suggested that in Col-*FRI*-Sf2 RNAi plants, with down-regulation of EARLI1-like genes, the synthesis of lignin was reduced and the proportion of monolignols was changed simultaneously, indicating that EARLI1-like genes might be involved in regulation of lignin synthesis. In semi-thin sections of root tips from 8-week-old RNAi plants stained with toluidine blue, the wall of most cells was thin and only five to 10 cells in the protoxylem were lignified, while the walls of 20–30 cells in protoxylem of wild-type Col-*FRI*-Sf2 plants at the same growth stage had been thickened by lignification (**Fig. 4D–E**). In order to more accurately examine the effect of EARLI1-like genes on lignin synthesis, the lignin content of 8-week-old Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants was quantified. The lignin content of RNAi plants (8.76% of stem dry weight) was lower than that of the wild-type Col-*FRI*-Sf2 plants (9.67% of stem dry weight) (**Fig. 5**) and the difference was significant at the $P < 0.05$ level. The decrease of ligin content in RNAi lines was consistent with histological observations.

In *Arabidopsis*, lignification mainly occurred in inflorescence stems after bolting. Because Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants flowered at different times, it was difficult to measure stem lignin content in the plant at different development stages. In our experiments, the lignin content of lignified stems of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants was determined after 8 weeks of growth. At this stage, the lignification process was close to completion and the data obtained could reflect a difference of lignification degree more precisely.

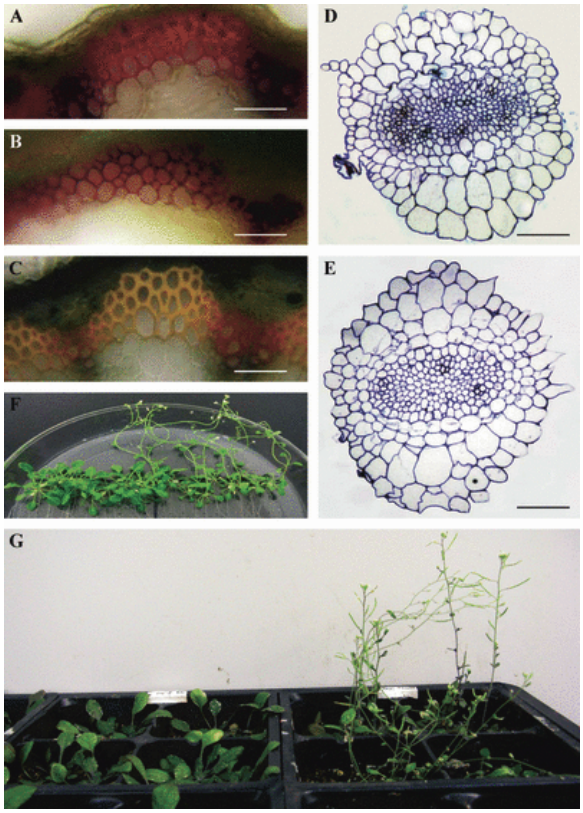

Figure 4 Influence of EARLI1-like genes on lignin synthesis. A, Stem section of 8-week-old Col-*FRI*-Sf2 plants stained with phloroglucinol, bar = 70 µm; B, C, Stem section of 8-week-old Col-*FRI*-Sf2 RNAi plants stained with phloroglucinol, bar = 70 µm; D, Root tip section of 8-week-old Col-*FRI*-Sf2 plant stained with toluidine blue, bar = 110 µm; E, Root tip section of 8-week-old Col-*FRI*-Sf2 RNAi plant stained with toluidine Blue, bar = 110 µm; F-G, Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants grown for 4 weeks in a long-day photoperiod, left are Col-*FRI*-Sf2, right are Col-*FRI*-Sf2 RNAi plants.

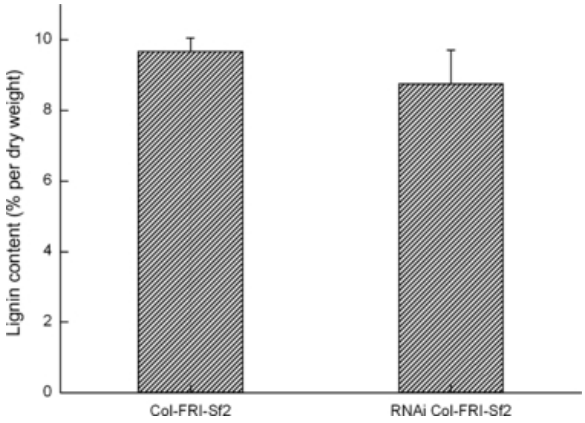

Figure 5 Determination of lignin content of inflorescence stems of 8-week-old plants. The data represent the average value of three independent RNAi lines. For each line, the experiments were repeated three times and ten plants were analysed at each time.

Cinnamoyl-CoA reductase (CCR) plays an important role in regulation of lignin synthesis in *Arabidopsis*, and is involved in the penultimate reductive reaction of monolignol synthesis. In order to determine the influence of EARLI1-like genes on lignin accumulation, expression of *CCR1* in inflorescence stems of Col-*FRI*-Sf2 and Col-*FRI*-Sf2 RNAi plants was monitored. RT-PCR analysis showed that the abundance of *CCR1* mRNA also declined in Col-*FRI*-Sf2 RNAi lines of *EARLI1* (**Fig. 6**). It is possible that EARLI1-like genes could affect expression of *CCR1*, resulting in a reduction of monolignol synthesis and final lignin content.

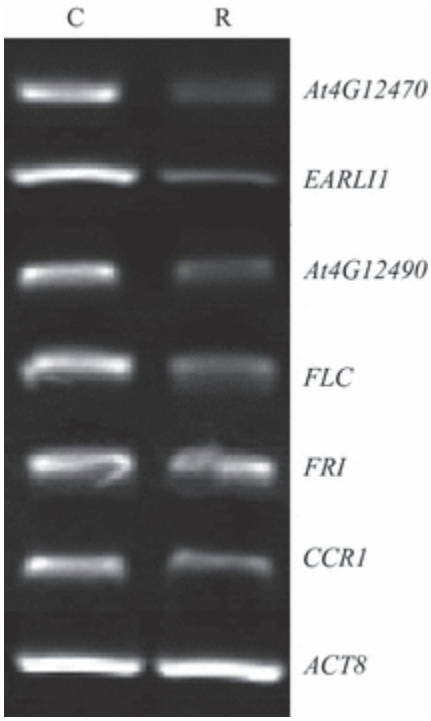

Figure 6 Expression analyses of flowering-related genes with RT-PCR. C, Col-*FRI*-Sf2; R, Col-*FRI*-Sf2 RNAi.

Col-*FRI*-Sf2 RNAi lines of *EARLI1* flowered earlier in long-day conditions

Under long-day photoperiods, Col-*FRI*-Sf2 RNAi plants of *EARLI1* flowered much earlier than wild-type Col-*FRI*-Sf2 plants. The time for RNAi lines and wild-type plants to bolting was 19.4 and 39.7 days, respectively (**Fig. 4F and G**; **Table 1**). Statistical analysis of various phenotype indices related to flowering showed that the length of the main inflorescence stem, number of internodes and number of rosette leaves in Col-*FRI*-Sf2 RNAi lines were much lower than in wild-type Col-*FRI*-Sf2 plants. These three indices of Col-*FRI*-Sf2 RNAi plants were 5.9, 4.6 and 6.8 cm, while in wild-type plants they were 14.8, 14.1 and 29.1 cm, respectively. We also examined the flowering time of Col-*FRI*-Sf2 RNAi plants under short-day conditions. In short days, the Col-*FRI*-Sf2 RNAi plants need about 35 days (with 25–30 rosette leaves) to flower, while Col-*FRI*-Sf2 plants bolted 2 months later and had 55–70 rosette leaves.

Table 1. Statistical analyses of phenotype indices.

ecotype	**Col-*FRI*-Sf2**	**Col-*FRI*-Sf2 RNAi**
length of main stem (cm)	14.8 ± 2.84a	5.9 ± 1.79b
number of internodes	14.1 ± 3.78a	4.6 ± 0.96b
number of lateral stems	2.1 ± 1.66a	1.9 ± 0.87a
number of rosette leaves	29.1 ± 6.57a	6.8 ± 0.91b
number of cauline leaves	7.7 ± 5.35a	6.3 ± 1.15b
time required for flowering (day)	39.7 ± 4.57a	19.4 ± 2.27b
time required for flowering (day) in SD	61 ± 3.79a	35 ± 3.23b

SD = short-day photoperiod.

Unless otherwise stated, the data represents the average value of three independent RNAi lines grown in long-day photoperiods. For each line, the experiments were repeated for three times and ten plants were analysed at each time. Data associated with a different letter in each column are significantly different according to Student's *t*-test ($P < 0.05$).

RT-PCR analysis revealed that expression of *FLC* in Col-*FRI*-Sf2 RNAi plants of *EARLI1* was repressed significantly, which might be the reason for early flowering. *FLC* is a core transcription factor of flowering regulation in *Arabidopsis*, and is related to many early flowering phenomena. Col-*FRI*-Sf2 is a late-flowering ecotype containing *FRI*, a gene involved in positive regulation of *FLC* in the vernalization flowering pathway of *Arabidopsis*, and activation of *FLC* by *FRI* is the reason for delayed flowering of this ecotype. In the present work, down-regulation of *EARLI1*, an *Arabidopsis* gene related to the vernalization pathway, resulted in earlier flowering in the *FRI* background. In order to determine whether the earlier flowering phenotype of Col-*FRI*-Sf2 RNAi lines of *EARLI1* was related to *FLC* and *FRI*, expression of these genes in 2-week-old RNAi and wild-type plants grown in long-day conditions was analysed. As shown in **Fig. 6**, along with repression of EARLI1-like genes, the expression of *FLC* also decreased remarkably, indicating that inhibition of *FLC* was an important factor in earlier flowering. However, as a positive regulator of *FLC*, the transcription of *FRI* showed no obvious change. Moreover, the expression of *SOC1* and *FT* was up-regulated (**Fig. 7**) in Col-*FRI*-Sf2 RNAi plants, indicating that RNA interference of EARLI1-like genes resulted in down-regulation of *FLC* and activation of *SOC1* and *FT*. Taken together, it is possible that knockdown of EARLI1-like genes might lead to earlier flowering through regulation of autonomous or long-day photoperiod pathways.

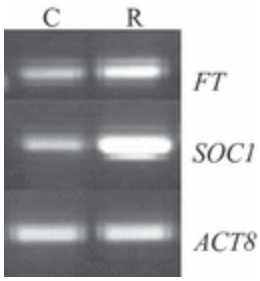

Figure 7 Expression analyses of *SOC1* and *FT* with RT-PCR. C, Col-*FRI*-Sf2; R, Col-*FRI*-Sf2 RNAi.

Discussion

Relationship of lignin synthesis and *EARLI1*

Lignin is mainly made up of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units derived from the plant phenylpropanoid pathway, and not only plays critical roles in maintenance of the structure of cell wall and enhancement of the mechanical strength of plant tissue, but also acts as a physical barrier in combination with cellulose for retention of water and resistance to biotic and abiotic stress (**Baucher *et al.* 1998**; **Bonawitz & Chapple 2010**). Cinnamoyl-CoA reductase (CCR) is a key enzyme in lignin biosynthesis that catalyses the penultimate step in production of monolignols. Down-regulation of CCR with transgenic approaches could modify the ultrastructure and mechanical properties of the cell wall (**Bjurhager *et al.* 2010**). In *Populus*, a decrease of CCR activity led to concentric sub-layering, disorganised architecture and colouration of the outer xylem (**Lepl*eet al.* 2007**). In *Arabidopsis*, limited CCR activity also resulted in a looser structure, disordered cellulose microfibril organisation, and thinner cell walls were observed in fibres and vessels (**Ruel *et al.* 2009**). Introduction of antisense *CCR* in tobacco leads to decreased lignin content and induces other biochemical changes involving polysaccharides, phenolic components of the cell wall and also soluble phenolics (**Chabannes *et al.* 2001**). Plants transformed with a vector containing a full-length *AtCCR1* cDNA in an antisense orientation showed a 50% decrease in lignin content, accompanied by changes in lignin composition and structure, with incorporation of ferulic acid into the cell wall. In these plants, microscopic analyses coupled with immunolabelling revealed a decrease in lignin deposition in normally lignified tissues and a dramatic loosening of the secondary cell wall of interfascicular fibres and vessels (**Goujon *et al.* 2003**).

CCR and cinnamyl alcohol dehydrogenase (CAD) catalyse the last steps of monolignol biosynthesis. Simultaneous repression of CCR and CAD results in dwarfism, reduced lignin content and abnormal lignin structure in *Arabidopsis thaliana*. Due to the lack of lignification in the anther endothecium, which is associated with anther dehiscence and pollen release, the triple *cad c cad d ccr1* mutant was male sterile. This suggests that CCR and CAD activities not only had impacts on lignification, but also on plant development (**Thévenin *et al.* 2010**). Similar phenotypes were reported in *Arabidopsis* mutants with a T-DNA insertion in the *CCR1* gene, as well as the hypomorphic mutant *irx4* with irregular xylem. T-DNA knockout mutants of *CCR1* had a dwarf phenotype and delayed senescence. At complete maturity, their inflorescence stems display a 25–35% decrease in lignin, some alterations in lignin structure, with a higher frequency of resistant inter-unit bonds and a higher content of cell wall-bound ferulic esters (**Mir Derikvand *et al.* 2008**). The *irx4* plants have 50% less lignin and a collapsed xylem phenotype (**Jones *et al.* 2001**). At the molecular level, plants with CCR deficiency have been shown to produce lignin that contains increased levels of ferulic acid (**Ralph *et al.* 2008**).

In the present work, we found EARLI1-like genes could influence the structure of leaf cells of *Arabidopsis*. Leaf cells of Col-*FRI*-Sf2 RNAi plants were comparatively long and thin in comparison with regular leaf cells of wild-type plants. In addition, EARLI1-like genes could influence the development of leaf trichomes; the base of trichomes in Col-*FRI*-Sf2 RNAi leaves was different from that of wild-type Col-*FRI*-Sf2 plants. Some trichomes in the RNAi leaf had irregular serrate base, while the base of wild-type trichomes was round. In roots of 8-week-old Col-*FRI*-Sf2 plants, cells with a thickened wall through lignification were very common, but the number of cells with thickened walls through lignification was obviously reduced in RNAi lines. During the same period, the cell wall in inflorescence stems was also thickened by lignification, and staining for lignin with phloroglucinol showed that the cell wall in the stems of Col-*FRI*-Sf2 RNAi plants was thin and some samples were dyed orange, while the wall of stem cells in wild-type Col-*FRI*-Sf2 plants was thicker and dyed red. **Besseau *et al.* (2007)** found that the cell wall was dyed orange with phloroglucinol and lignin synthesis was decreased in inflorescence stems when activity of hydroxycinnamoyl-CoA:shikimate/quinate hydroxy-cinnamoyltransferase (HCT) was down-regulated, and the reduction of syringyl lignin and guaiacyl lignin was especially striking, from 80% and 15% to 11% and 5%, respectively. Colour changes of lignin in phloroglucinol staining in *EARLI1* RNAi lines was probably also related to decreased HCT expression and decreased syringyl lignin and guaiacyl lignin content. Quantitative analyses revealed the lignin content in inflorescence stems of Col-*FRI*-Sf2 RNAi plants was lower than that of wild-type Col-*FRI*-Sf2 plants and accounted for 8.76% and 9.67% of stem dry weight, respectively. RT-PCR analyses showed that expression of EARLI1-like genes was positively related to expression of *CCR1* encoding a key enzyme in lignin synthesis. According to the above experimental results, we deduced that EARLI1-like genes might regulate the biosynthesis of monolignols indirectly through the influence on *CCR1* and ultimately affect lignin content.

Relationship of *EARLI1* and flowering time

Genetic analyses using *Arabidopsis* have revealed that flowering is mainly regulated by photoperiod-, autonomous-, vernalization- and gibberellin-induced pathways (**Bäurle & Dean 2006**). In general, the floral induction signals from different pathways will be transmitted and will converge to two central regulators, *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*). *CO* acts as a floral activator and mediates the photoperiod pathway, whereas *FLC* acts as a floral repressor and mediates the autonomous and vernalization pathways. *CO* is responsible for activation of *FLOWERING LOCUS T* (*FT*), while *FLC* is involved in repression of *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (**Seo *et al.* 2009**). Both *FT* and *SOC1* are associated with determination of flowering time, but *FT* is the major output of *CO*. *SOC1* is regulated by *CO* through *FT* (**Yoo *et al.* 2005**; **Xi & Yu 2009**), and integrates the photoperiod pathway also through *FT*. FT is a mobile signal transmitted from the leaf to the meristem to initiate flowering under long-day photoperiods (**Jang *et al.* 2009**). Because the activation of *FT* by *CO* occurs specifically in the phloem, the 20 kDa FT protein must move to the shoot apical meristem (SAM) to become functional (**Searle *et al.* 2006**). FLC can repress the expression of *FT* and *SOC1* directly by binding to the promoters of *SOC1* and the first intron of *FT* (**Searle *et al.* 2006**). The autonomous and vernalization pathways promote flowering by repressing *FLC* expression (**Bäurle & Dean 2006**). In conclusion, the floral meristem identity gene *LFY* (*LEAFY*) activated by SOC1 and AGL24 and *AP1* (*APETALA1*) activated by FT would promote flower development at the inception of the shoot apical meristem (**Lee & Lee 2010**).

FLC is a key repressor of flowering in *Arabidopsis*, and its expression is regulated both positively and negatively by post-translational histone modifications. Vernalization epigenetically silences *FLC* expression through H3K9me2 and H3K27me3 dimethylation, while RNA polymerase II-associated complex Paf1c activates *FLC* expression through increased H3K4 and H3K36 methylation. The mutation effects of At3g22590, an *Arabidopsis* homologue of the yeast Paf1c component CDC73, are primarily limited to flowering time. The *cdc73* mutants show reduced *FLC* mRNA levels and decreased H3K4me3 at the *FLC* locus (**Yu & Michaels 2010**). Apart from histone modification, expression of *FLC* could also be controlled by other genes and by antisense RNA. Epistasis analyses suggest that EDM2 acts upstream of the floral repressor FLC in a regulatory module that resembles the autonomous floral promotion pathway and affects the floral transition by regulating *FLC* transcript levels (**Tsuchiya & Eulgem 2010**). Two *Arabidopsis thaliana* components of the autonomous flowering pathway, FCA and FPA, appear to control *FLC* transcription by mediating alternative polyadenylation of embedded non-coding antisense RNAs (**Hornyik *et al.* 2010**). CstF64 and CstF77 are required for 3' processing of *FLC* antisense transcripts, and can trigger localised histone demethylase activity and result in reduced *FLC* sense transcription (**Liu *et al.* 2010**).

FLC and *FRIGIDA* (*FRI*) are core members of the vernalization pathway. Most winter ecotypes of *Arabidopsis* such as Stokholm and San Felieu2 have functional *FLC* and *FRI*, while summer ecotypes of *Arabidopsis* have a non-functional *FRI* and/or a dysfunctional *FLC* (**Michaels *et al.* 2003**). Over-expression of *FLC* in winter ecotypes of *Arabidopsis* leads to a serious late-flowering phenotype. In contrast, inhibition of *FLC* expression results in earlier flowering. *FRI* can repress the transition process of vegetative growth to reproductive stage by promotion of *FLC* expression, while vernalizaton can counteract the effect of *FRI*, inhibit the expression of *FLC* and induce plants to flower in spring of the next year (**Johanson *et al.* 2000**).

In Col-*FRI*-Sf2 RNAi plants grown in long-day conditions, expression of EARLI1-like genes was remarkably inhibited. Because the RNA interference construct was designed based on the conservative 8CM motif of *EARLI1*, the expression of *At4G12470* and *At4G12490* was also repressed, but the silenceing degree was relatively mild. This result was probably derived from differences between nucleotide sequences of three genes. Our experiments confirmed that EARLI1-like genes could be effectively down-regulated by RNA interference.

In plants, the transition from vegetative to reproductive stage is a complex and accurate regulation process, and flowering can be considered as a marker of the end of the vegetative growth and beginning of reproductive growth. In *Arabidopsis*, the time required for flowering represents the length of the vegetative growth time, and new rosette leaves will constantly appear in this period. The number of rosette leaves can reflect the length of vegetative growth, while the length of the main stem, number of internodes, number of cauline leaves and number of lateral stems reflect the length and status of reproductive growth. Statistical analyses showed that the vegetative period and reproductive stage of Col-*FRI*-Sf2 RNAi plants were much shortened, and the time required for flowering, number of rosette leaves, length of main stems and number of internode were all less than in wild-type Col-*FRI*-Sf2 plants. This suggests that EARLI1-like genes could affect the whole process of *Arabidopsis* growth. In order to further determine the molecular mechanism of the inhibition effect of EARLI1-like genes on flowering, expression of several genes in vernalization, autonomous and long-

day photoperiod pathways was analysed in the present work. The results showed that at the same time as silencing of *At4G12470*, *EARLI1* and *At4G12490*, transcription of *FLC* also declined remarkably, but expression of *FRI* was not changed. In addition, two central regulators of flowering time, *SOC1* and *FT*, were activated. Because the material used in this work was not subjected to vernalization treatment, the reduction of *FLC* expression and the earlier flowering phenomena in *EARLI1* RNAi plants probably came from another pathway than the vernalization pathway. It is possible that *EARLI1* can promote *FLC* expression and postpone flowering time by its regulation function on critical genes in autonomous pathway or long-day photoperiod pathway. Once the expression of *EARLI1* is silenced, the inhibition effects of transcriptional factors in the autonomous or long-day photoperiod pathway to *FLC* would be activated, and a decrease of *FLC* expression would activate *SOC1* and *FT*, leading to early flowering. Because the experiments were carried out in a long-day photoperiod that could promote flowering independent of *FLC*, it is possible that EARLI1-like genes are also involved in the long-day photoperiod pathway of flowering, which might have synergistic or overlapping effects with the *FLC*-dependent flowering process.

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