RESEARCH PAPER

Influence of EARLI1-like genes on flowering time and lignin synthesis of Arabidopsis thaliana

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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 ABL, 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 (José-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 harbouiring 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...
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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 synthesis 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% HgCl2, 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 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/(N-morpholino) propanesulfonic 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′-TTTCTTCGCCCTTTAACATCA-3′ and 5′-AA-GCCAGACGGAAACCTTTTC-3′; PCR primers for At4G12470 were 5′-GCTTCAAAGAACTCAGCCTC-3′ and 5′-TAC-AAAGGTTGAGGGGC-3′. The probe for At4G12490 was prepared with PCR primer pair 5′-CCATTTGCCCACTACAAT-3′ and 5′-GCAATTGCGACATAACC-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 detection. 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 the instructions for the Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesised with the RevertAid First Strand cDNA Synthesis kit kit #1621 (Fermentas, Burlington, ON, Canada). RT-PCR primers for CRR1 were 5′-TTCTCCTGAGTGGCCTCTCA-3′ and 5′-AG-GTTCTGTCGGTCCTGC-3′. RT-PCR primers for ACT8 were 5′-ATGAAGATATTAAAGTGGTGCA-3′ and 5′-TCC-GAGTTGGAAGAGGTAC-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′-GACATTGCCGCCTGGTTCAT-3′ and 5′-AGGCGAGTCGAGGAG-TGTTCC-3′; for FRI were 5′-TTGAAGAAAGTCTGCG-TAACG-3′ and 5′-CACCTTGCTTATTACAGAT-3′; for SOCI were 5′-CCCTTGAATGCTCATGTC-3′ and 5′-CTTGGG-CTACTTCTTCATC-3′; and for FT were 5′-GGTTGTTGAGACGTTCCTTG-3′ and 5′-CCTCCGACGACACTCTCCC-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 plants 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% OsO4 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 CO₂, 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 N₂ 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.

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 cuticles 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).

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

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**Fig. 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.
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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 and FRI 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 FRI 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 lignin 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.

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.

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 bolted 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.

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...
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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 (Leplé et 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 num-

![Fig. 3. SEM and phenotype observations of Col-FRI-Sf2 and Col-FRI-Sf2 RNAi plants.](image)

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.
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Fig. 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.

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 (Baurle & 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 CONSTANS (SOC1) (Searle 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 (Baurle & Dean 2006). In conclusion, the floral meristem identity gene LFY (LEAFY) activated by SOC1 and AGL24 and API (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 H3K6 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). Overexpression 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 inhibition of FLC expression, while vernalization 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 silencing 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

**Table 1.** Statistical analyses of phenotype indices.

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

**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).
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, SOCI 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 SOCI 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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