Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering


A main developmental switch in the life cycle of a flowering plant is the transition from vegetative to reproductive growth. In Arabidopsis thaliana, distinct genetic pathways regulate the timing of this transition. We report here that brassinosteroid (BR) signaling establishes an unexpected and previously unidentified genetic pathway in the floral-regulating network. We isolated two alleles of brassinosteroid-insensitive 1 (bri1) as enhancers of the late-flowering autonomous-pathway mutant luminidependens (ld). bri1 was found to predominantly function as a flowering-time enhancer. Further analyses of double mutants between bri1 and known flowering-time mutants revealed that bri1 also enhances the phenotype of the autonomous mutant fca and of the dominant FRI line. Moreover, all of these double mutants exhibited elevated expression of the potent floral repressor FLOWERING LOCUS C (FLC). This molecular response could be efficiently suppressed by vernalization, leading to accelerated flowering. Additionally, specific reduction of the expression of FLC via RNA interference accelerated flowering in bri1 ld double mutants. Importantly, combining the BR-deficient mutant cpd with ld also resulted in delayed flowering and led to elevated FLC expression. Finally, we found increased histone H3 acetylation at FLC chromatin in bri1 ld mutants, as compared with ld single mutants. In conclusion, we propose that BR signaling acts to repress FLC expression, particularly in genetic situations, with, for example, dominant FRI alleles or autonomous-pathway mutants, in which FLC is activated.

KEY WORDS: Brassinosteroid, Flowering time, BR1, FLC, Autonomous pathway, luminidependens, Arabidopsis thaliana

INTRODUCTION

A major developmental transition in plants is the switch from the vegetative to the reproductive phase. Timing this transition, such that it occurs under the most advantageous conditions for pollination and seed production, is essential to maximize reproductive success. In Arabidopsis thaliana, flowering time is controlled by several pathways, which integrate environmental signals with the developmental status of a plant (reviewed in Boss et al., 2004; Komeda, 2004; Putterill et al., 2004). Genetic analysis of late-flowering mutants identified the photoperiod, the gibberellin, the vernalization pathways, which integrate environmental signals with the transition from the vegetative to the reproductive phase (Boss et al., 2004; Henderson and Dean, 2004; Komeda, 2004). These multiple floral-promoting signals regulate expression of a common set of genes collectively termed floral-pathway integrators. FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), also known as AGL20 – The Arabidopsis Information Resource (TAIR) – and LEAFY (LFY) were shown to function at this convergence point (Nilsson et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999; Blazquez and Weigel, 2000; Lee et al., 2000; Samach et al., 2000; Hepworth et al., 2002; Moon et al., 2003). Floral-pathway integrators activate floral-meristem identity genes and these trigger the transition from the vegetative to the reproductive phase (Boss et al., 2004; Henderson and Dean, 2004; Komeda, 2004).

The autonomous pathway constitutes a heterogeneous group of genes that includes FVE, FLOWERING LOCUS D (FLD), LUMINIDEPENDENS (LD), FLOWERING LOCUS K (FLK), FY, FCA and FP4 (Koornneef et al., 1991; Koornneef et al., 1998). Gibberellin biosynthesis and signaling mutants are markedly delayed in floral transition under non-inductive short days (Wilson et al., 1992; Jacobsen and Olszewski, 1993). The autonomous pathway was defined based on the behavior of mutants that display photoperiod-independent late flowering and strong acceleration of flowering in response to prolonged exposure to cold (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991). Lastly, the vernalization pathway represents the promoting activity of prolonged cold treatment as it naturally occurs in winter (Chouard, 1960; Lang, 1965). These multiple floral-promoting signals regulate expression of a common set of genes collectively termed floral-pathway integrators. FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), also known as AGL20 – The Arabidopsis Information Resource (TAIR) – and LEAFY (LFY) were shown to function at this convergence point (Nilsson et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999; Blazquez and Weigel, 2000; Lee et al., 2000; Samach et al., 2000; Hepworth et al., 2002; Moon et al., 2003). Floral-pathway integrators activate floral-meristem identity genes and these trigger the transition from the vegetative to the reproductive phase (Boss et al., 2004; Henderson and Dean, 2004; Komeda, 2004).

FLC encodes a MADS-domain transcription factor that quantitatively represses flowering (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001). FLC antagonizes the activity of floral-promoting pathways, at least partly, by directly binding to specific regulatory elements in the FT and SOC1 loci (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006). Additionally, FLC works together with FRIGIDA (FRI) as the major determinants of vernalization requirement in Arabidopsis thaliana (Napp-Zinn, 1961; Michaels and Amasino, 1999; Sheldon et al., 2000). FRI functions via an unknown biochemical mechanism to transcriptionally upregulate FLC expression to levels that override

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the effects of floral-inducing signals (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000; Michaels and Amasino, 2001). Thus, FRI-harboring A. thaliana accessions with a functional FLC phenotypically resemble autonomous mutants (Michaels and Amasino, 1999). The late flowering of FRI and autonomous mutants is suppressed by vernalization treatment (Napp-Zinn, 1961; Koornneef et al., 1998), which quantitatively accelerates flowering by stably repressing FLC expression (Michaels and Amasino, 1999; Sheldon et al., 1999).

The regulation of chromatin structure via diverse histone modifications has recently been reported as a crucial molecular mechanism in the control of FLC expression (reviewed in He and Amasino, 2005). Histone acetylation and trimethylation at lysine 4 of histone 3 (triMeH3K4) were found to be correlated with active transcription of FLC (He et al., 2003; Ausin et al., 2004; He et al., 2004; Kim et al., 2005). The enrichment in the triMeH3K4 histone mark depends on the activity of the PAF1 complex, which, in A. thaliana, consists of EARLY FLOWERING 7 (ELF7), ELF8, VERNALIZATION INDEPENDENCE 4 (VIP4) and VIP5 (He et al., 2004; Oh et al., 2004). The PAF1 complex is required for FLC expression both in FRI-containing lines and in autonomous mutants. Based on the role of the yeast PAF1 complex, it has been speculated that the A. thaliana complex associates with RNA polymerase II and recruits a H3K4 methyltransferase to the actively transcribed regions (He et al., 2004; Oh et al., 2004). EARLY FLOWERING IN SHORT DAYS (EFS), a putative histone H3 methyltransferase, was also shown to be required for FLC expression and for triMeH3K4 (Kim et al., 2005). Finally, EARLY FLOWERING IN SHORT DAYS 1 (ESD1; also known as ACTIN-RELATED PROTEIN6) (ARP6) and ATARP6 – TAIR was recently described above are, at least partly, due to impaired BR-signaling. Finally, histone H3 acetylation at the FLC locus was found to be enriched in bri1 ld double mutants, compared with ld single mutants, and this was associated with enhanced FLC expression in this double mutant.

**MATERIALS AND METHODS**

**Plant material**

All experiments were carried out using Arabidopsis thaliana ecotype Wassilewskija-2, termed in the paper Ws. ld-2 (Lee et al., 1994) and bri1-4 (Noguchi et al., 1999) were obtained from the NASC stock center. FRI is a Ws line with the FRI allele from San Feliz-2 (SF2) (Lee et al., 1993). gi-1 was provided by J. Putterill (Fowler et al., 1999) and cpd–39.9 was a gift from F. Tax (Noguchi et al., 2000). The gal-3 mutant, provided by S. Bednarek (University of Wisconsin-Madison, Madison, WI), originally in the Ler background (Sun and Kamiya, 1994), was introgressed into Ws through three recurrent backcrosses. Double mutants were obtained from respective crosses by identifying homozygous ld, FRI, fca, gi or gal mutants segregating the bri1 mutation. Homozygous lines were selected based on late flowering, gibberellin (GA) deficiency or by using molecular markers (Johanson et al., 2000).

**Isolation of enhancers of ld-3**

ld-3 (Lee et al., 1994) was mutagenized with ethylmethane sulfonate, according to standard practices. From the resultant collection of M2 plants grown under continuous light, three extremely late-flowering plants were selected and, of these, two survived and were recovered. Both of these ld-3 modifiers were mapped to the BRI1 locus and are here termed bri1-201 and bri1-202. These lines were backcrossed to the Ws wild type to reduce the mutagenesis load, and to select the bri1-201 and bri1-202 single mutants.

**Construction of FLC-RNAi bri1-201 ld-3 lines**

The 5’ UTR region of the FLC transcript was amplified with 5’-GGGG-attB1-CCCGAGAAAAAGGAAAAAATTTAAA-3’ and 5’-GGGGAattB2-CGGCTTCTTCTCGAGGAGG-3’ primers and cloned into the pDONR207 vector using the GATEWAY system (Invitrogen, Karlsruhe, Germany). Subsequently, the cloned FLC fragment was inserted as two inverted copies into the plant-transformation vector p Jawohi8-RNAi (provided by B. Uilker and Dr I. Somsich, MPIZ, Cologne, Germany). The resulting construct was introduced into the bri1-201 ld-3 double mutants by the floral-dip method (Clough and Bent, 1998).

**Growth conditions and flowering-time measurements**

Seeds were stratified for 2-5 days at 4°C in darkness on half-strength (2.2 g/l pH 5.7) MS-medium (Murashige and Skoog, 1962) (Sigma-Aldrich, Taufkirchen, Germany), with 1.2% (w/v) agar prior to transferring to soil. Plants were grown in a controlled environment cabinet under long or short days, as described (Reeves et al., 2002). For vernalization treatment, stratification was followed by incubation for 2 days at 22°C under a photoperiod of 12 hours of light/12 hours of darkness, in order to induce synchronized germination. Germinated seeds were returned to 4°C for 6 weeks under a short-day photoperiod (8 hours of light/16 hours of darkness). Flowering time was scored as the number of rosette leaves at flowering when the bolt was approximately 1 cm high. A total of 10-18 plants per genotype were analyzed in each experiment. Data are expressed as mean±s.e.m.
**Analysis of FLC mRNA abundance**

Tissue was harvested from the aerial parts of plants 9 hours after dawn. Total RNA was isolated from the plants using the Plant RNeasy kit (Qiagen, Hilden, Germany). RNA (7.5-15 μg) was separated on 1.5% agarose gels. The DNA was stained with ethidium bromide and visualized using a PhosphorImager (Molecular Dynamics, USA). Analysis of FLC mRNA abundance was performed as described (Reeves et al., 2002). An ACTIN 1 (ACT1) fragment was amplified by PCR for use as a probe. The ACT1 primers used were: 5'-TGCGACAATGGAACTGGAATG-3' and 5'-AAAATCTCTTTGGCTACCATCG-3'. Hybridization was performed according to Sambrook and Russell (Sambrook and Russell, 2001). The bands were visualized using a PhosphorImager (Molecular Dynamics, USA) and signal strengths were quantified using its ImageQuant software.

**Analysis of FT and SOC1 mRNA abundance**

FT and SOC1 expression was analyzed by reverse transcriptase (RT)-PCR. Total RNA (2-2.5 μg) isolated from the plants using the Plant RNeasy kit (Qiagen, Hilden, Germany) was used for cDNA synthesis. Prior to this synthesis, RNA was treated with DNase I (Roche, Mannheim, Germany). Synthesized cDNA (20 μl) was diluted with H2O to a final volume of 100 μl prior to PCR. PCR was carried out in a total volume of 20 μl; 2 μl of cDNA was used per 20 μl of reaction volume. Primers to amplify FT and SOC1 were as described (Searle et al., 2006). A total of 27 and 24 PCR cycles were applied, respectively. 22 PCR cycles were used to amplify UBQ10 (5'-AACT-3' and 5'-CACAACATGGCAATAGCTCAA-3'). Hybridization was performed according to Sambrook and Russell (Sambrook and Russell, 2001). The bands were visualized using a PhosphorImager (Molecular Dynamics, USA) and signal strengths were quantified using its ImageQuant software.

**ChIP assays**

Chromatin immunoprecipitation (ChIP) assays were performed as described (Searle et al., 2006), using 21-day-old Ws, bri1-201, ld-3 and bri1-201 ld-3 grown under the same long-day conditions as was described for the flowering-time experiments. ChIP used antibodies against acetylated histone H3 or against trimethylated histone H3 at lysine 4 (06-599 and 07-473, respectively, Upstate Biotechnology). As a negative control, antibodies against anti-rat-IgG (AB6703, Abcam) were used. DNA was dissolved in 100 μl 10 mM Tris-HCl, pH 7.4. DNA (2 μl) was used in PCR at a total volume of 20 μl. To amplify regions I and II of the FLC locus, the following pairs of primers were used: 5'-GCCACATGCGCTACCATGAC-3', 5'-CCCAAATCTTTTTGCTAATCG-3' and 5'-TTGTTACATTCTCA-AACGGTATAATCT-3'. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, visualized and quantified using a PhosphorImager (Molecular Dynamics, USA). Two independent biological replicates and two independent chromatin immunoprecipitations were analyzed. The PCR products were separated on 2% agarose gel, stained with ethidium bromide, visualized and quantified using a PhosphorImager (Molecular Dynamics, USA).

**RESULTS**

**bri1 is a strong enhancer of the autonomous mutant ld**

In an attempt to identify additional flowering-time regulators, we performed a genetic screen in which we mutagenized the autonomous mutant luminindependens (ld) and isolated enhancers of its late-flowering phenotype. Two recessive allelic mutations were isolated, which, upon detailed examination, were found to extend the ld phenotype to extremely late flowering (Fig. 1A,B). Both mutations were mapped to the BR1 locus. The isolated alleles, bri1-201 and bri1-202, were found to have point mutations that affect the encoded BR-binding domain and the kinase domain, respectively (Fig. 1C). Both single bri1 mutant alleles flowered much earlier than the single ld mutant (13 compared with 25 rosette leaves), and only combining them with ld resulted in severe late flowering (Fig. 1A,B). We confirmed the extremely late-flowering phenotype of the bri1 ld double mutants by reconstituting the phenotype with alternative alleles of bri1 and/or ld. The flowering time of all combinations of the double bri1 ld mutants compared to the photoinduced phenotypes in the...
original double mutants found in the enhancer screen (Fig. 1D,E). The fact that all bri1 alleles tested delayed both ld alleles implies that the extent of this flowering effect was neither ld-allele specific nor bri1-allele specific.

**bri1 is a strong enhancer of FRI and autonomous mutants**

To further characterize the role of BRI1, we analyzed the floral timing of double mutants of bri1 and known flowering-time mutants. For this study, we chose another autonomous- (fca-11), a photoperiod- (gi-11) and a gibberellin- (ga1-3) pathway mutant, and dominant FRI in the Ws background. Because all bri1 alleles tested had a comparable effect on the flowering of ld mutants, we selected the bri1-201 allele for further double-mutant analyses. Under long days, double bri1 fca, bri1 FRI and bri1 ld mutants exhibited a similar extremely late-flowering phenotype (approximately 66, 64 and 62 rosette leaves, respectively) compared with the single fca, FRI and ld lines (approximately 29, 22 and 26 leaves, respectively; see Fig. 2A,B). The gi mutant, impaired in the photoperiod pathway, was the latest-flowering single mutant under long-day conditions, but the introduction of the bri1 mutation delayed flowering only modestly (around 49 versus 30 leaves in the gi single mutant), and this gi bri1 double mutant was not as late flowering as the double fca bri1, FRI bri1 nor ld bri1 mutants. The gibberellin-deficient ga1 mutant had a mild late-flowering phenotype (16.4 leaves) under long days, but
flowered later than the single bri1 mutant. The double ga1 bri1 mutant exhibited later flowering (23.8 leaves) than either single, but ga1 bri1 was still earlier flowering than all other analyzed double mutants with bri1 (Fig. 2). We also analyzed the double bri1 ld and the bri1 gi mutants under a non-inductive photoperiod. Similar to what we observed under long days, the single bri1 mutant had a mild late-flowering phenotype (approximately 34 leaves compared with 30 leaves of the wild-type control), and the double bri1 ld mutant was severely delayed in flowering compared with the single ld mutant (around 71 and 39 leaves, respectively, Fig. 2C). The single gi mutant was only slightly late flowering (approximately 36 leaves), and the double gi bri1 mutant flowered marginally later than either gi or bri1 single mutant; gi bri1 flowered much earlier than bri1 ld. We concluded that the BRI1 pathway has limited interaction with the photoperiod and the gibberellin pathways, and might function through the autonomous pathway.

**bri1 elevates FLC expression in FRI and in autonomous mutants**

Autonomous-pathway mutants and dominant FRI-harboring lines exhibit a similar late-flowering phenotype, which correlates with an elevated expression of a potent floral repressor, FLC (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001). Thus, if the BRI1-pathway interacts with the autonomous pathway to regulate flowering, we hypothesized that the combination of bri1 with autonomous-pathway mutants would result in altered levels of FLC mRNA. To address this, we investigated FLC expression in the bri1-201 ld-3 double mutant, in the single ld-3 and bri1-201 mutants, and in the controls (Fig. 3A,B). We monitored FLC levels by RNA-blot analysis throughout development (until flower buds were visible) in plants grown under long days. As expected, we detected only traces of FLC in the wild type, but the levels were quite high in the ld mutant. Interestingly, later during the life cycle of ld mutants, FLC expression decreased (Fig. 3A,B). This decrease correlated with its time of flowering. The bri1 mutant had low levels of FLC transcript, comparable to the levels in the Ws control. This is consistent with the modest flowering phenotype of this single mutant. In the bri1 ld double mutant, FLC transcript accumulated to much higher levels than in the single ld mutant (Fig. 3A,B). Moreover, levels of FLC remained very high throughout the assayed time-course (approximately two- to three-fold times higher than the highest levels in the ld mutant), even in plants that were around 100-days-old.

Because flowering in the bri1 ld mutant was also delayed compared with ld under short-day photoperiods, we assessed FLC expression under this condition. We analyzed 14- and 30-day-old plants, and detected elevated FLC levels in bri1-201 ld-3 and bri1-202 ld-3 mutants compared with the expression in ld-3 (after 14 days: 0.67 and 0.63, respectively, compared with 0.4; after 30 days: 1.00 and 0.83, respectively, versus 0.31; Fig. 3C).
We further tested FLC expression in other allele combinations of bri1 and ld. We chose to examine the expression after 30 days of growth, which was a time when the difference in FLC expression level was unambiguous between bri1-201 ld-3 double mutants and the single ld-3 mutant. Wild-type control plants were excluded from this experiment because they had already flowered. We observed elevated FLC expression in all tested bri1 ld double mutants (0.77, 0.67 and 0.68 compared with 0.27 in case of the ld-3 allele; and 0.73 and 1.0 compared with 0.28 for the ld-2 allele, relative to the maximal level detected; Fig. 3D).

The similar flowering phenotype of bri1 ld to bri1 fca and bri1 FRI led us to test whether bri1 also affects FLC expression in the fca mutant and FRI-bearing line. The 30-day-old double-mutant combinations between bri1-201 and fca or FRI, and the respective single mutants, were analyzed. The fca bri1 and FRI fca bri1 genotypes exhibited elevated expression of FLC compared with the single fca and FRI lines, respectively (0.42 versus 0.23 and 0.42 versus 0.13; Fig. 3E). At the same time, the presence of the bri1 mutation did not lead to an increase in FLC expression in the photoperiod mutant gi (Fig. 3E), further confirming that the enhancement effects of bri1 are specific to ‘high FLC-expressers’, such as FRI and autonomous mutants.

**Vernalization efficiently promotes flowering of bri1 ld, bri1 fca and bri1 FRI double mutants**

Prolonged exposure to cold (vernalization) is a well-described process that promotes flowering (Chouard, 1960; Lang, 1965). In particular, the late-flowering phenotype of plants that contain high levels of FLC (e.g. autonomous-pathway mutants and FRI) can be suppressed by a prolonged exposure to cold (Koornneef et al., 1991; Michaels and Amasino, 1999; Sheldon et al., 1999). Therefore, we expected that, if bri1 delays flowering of the tested autonomous mutants and FRI through enhancing FLC expression, then vernalization treatment would suppress the late-flowering phenotype of bri1 ld, bri1 fca and bri1 FRI. Indeed, the vernalized bri1 ld, bri1 fca and bri1 FRI mutants flowered almost at the same time as the single ld/fca/FRI mutants (Fig. 4A). Interestingly, single bri1 mutants responded only partially to vernalization (acceleration from approximately 13.5 rosette leaves to 10.5). We also investigated the effect of the prolonged exposure to cold on FLC mRNA abundance in the double bri1 ld/fca/FRI lines, compared to the respective single mutants (Fig. 4B,C). A clear repression of FLC expression was observed in all lines that exhibited high FLC levels before exposure to cold. Thus, reduction of FLC levels by vernalization efficiently suppresses the late-flowering phenotype of the double bri1 ld/ fca/FRI mutants.

**Reduction of FLC expression accelerates flowering of bri1 ld double mutants**

To confirm that high FLC expression is the major determinant of late flowering in double mutants of bri1 with autonomous-pathway mutants, we created an FLC-RNAi silencing construct, introduced it into the bri1-201 ld-3 double mutant and analyzed the flowering time of the resultant lines. The FLC-RNAi construct efficiently reduced expression of FLC in all ten transgenic lines analyzed, because these modified bri1 ld plants were found to have significantly lower levels of FLC transcript compared with the non-silenced plants harboring the control vector (Fig. 4F). Importantly, we did not observe any apparent decrease in the levels of two FLC-relatives - MAF1 and MAF5 – in the analyzed FLC-RNAi transgenic lines (data not shown), implying that the silencing construct specifically targets FLC mRNA. All plants harboring FLC-RNAi exhibited a pronounced acceleration of flowering compared with the control bri1 ld mutants (Fig. 4D,E). In conclusion, the marked effect of reduction of FLC expression on flowering time of double bri1 ld mutants provides the ultimate confirmation that the level of FLC plays a crucial role in delaying the flowering time of this double mutant.

**The BR-deficient mutant cpd enhances FLC expression in the ld background**

After identifying BR1 as an important modulator of flowering time, we wondered whether the observed effects reflect the role of BR1 in BR signaling. To address this, we examined whether the reduction in endogenous BRs leads to a similar phenotype as we found for the bri1 mutant. The BR-deficient mutant, constitutive
Photomorphogenesis and dwarfism (cpd), which is blocked at one of the last steps of BR biosynthesis (Szekeres et al., 1996), was chosen for these studies. The severity of the phenotype of cpd loss-of-function mutants is comparable to the phenotype of strong bri1 alleles (Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory, 1997). We first analyzed flowering time under a long-day photoperiod of the cpd-3939 loss-of-function allele, and compared it to bri1-201. Single cpd mutants exhibited a modest late-flowering phenotype, similar to bri1 (bolting after ~13 leaves), but, when flowering was measured as days to the start of bolting, cpd flowered later than bri1 (41 versus 33.8 days) (Fig. 5A,B). Introducing cpd into the ld-3 background led to markedly delayed flowering (Fig. 5A,B). When flowering time was measured as days to bolting, cpd ld mutants flowered later than bri1 ld (approximately 116 versus 94 days; Fig. 5A). By mild contrast, when counting rosette leaf number at flowering, the bri1 ld mutant was found to be more delayed in flowering than cpd ld (Fig. 5B). Because we observed that cpd enhances the late-flowering phenotype of ld mutants, we wondered whether elevated FLC mRNA levels could be detected. FLC mRNA levels were examined in 30-day-old cpd-3939 ld-3 double mutants, and this was compared to the respective single mutants and to the bri1-201 ld-3 double mutant, all grown under long days. As expected, we could not detect by RNA-blot analysis FLC transcript in the single cpd-3939 mutant. Importantly, we observed enhanced FLC expression in cpd ld mutants compared with ld (0.61 versus 0.27), and the FLC levels in cpd ld were comparable to that seen in bri1 ld (0.77) (Fig. 5C). We further tested whether increased expression of FLC resulted in down-regulation of its two direct targets, FT and SOC1 (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006). In the samples tested above, both FT and SOC1 levels were found to be lower in the double mutants cpd ld and bri1 ld than in the single ld mutant (Fig. 5D). As expected, cpd and bri1 single mutants expressed FT and SOC1 to higher levels than ld mutants or the respective double mutants. In summary, we observed that BR deficiency delays flowering of ld mutants and enhances FLC expression in the ld background in a similar manner as bri1. Thus, we concluded that a block in BR11-dependent BR signaling leads to a severe delay in flowering of the autonomous mutant ld, probably by elevating the levels of the FLC transcript, which, in turn, causes a decreased expression of the floral pathway integrators FT and SOC1.

**Compared with ld single mutants, increased histone H3 acetylation at FLC chromatin is found in bri1 ld double mutants**

Recent findings revealed the importance of modifications of chromatin structure at the FLC locus in the regulation of its expression (reviewed in He and Amasino, 2005). For example, histone 3 (H3) acetylation was shown to correlate with a transcriptionally active state, and histone triMeH3K4 is required for FLC expression (He et al., 2003; Ausin et al., 2004; He et al., 2004). Enrichment of triMeH3K4 at the FLC locus is required for high levels of expression both in the autonomous mutant and FRI backgrounds. Introduction of this histone modification depends on the activity of the PAF1 complex and a putative histone H3 methyl transferase, EFS (He et al., 2004; Oh et al., 2004; Kim et al., 2005). Because presence of the bri1 mutation enhances FLC expression in ld, fca and FRI mutants, we wondered whether it did so by affecting the levels of H3K4 trimethylation in FLC chromatin. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) with antibodies against triMeH3K4 histones. At 21-days old, Ws, bri1-201, ld-3, and the bri1-201 ld-3 double mutants grown under long days were examined and assayed at the FLC locus. In this experiment, we analyzed region IV of the locus (corresponding to the 5' UTR and the first exon, see Fig. 6A), because it was shown to be most enriched in this histone modification in high-FLC-expressing lines (He et al., 2004). As expected, we found strong increases in triMeH3K4 in ld mutants (Fig. 6B). We did not observe evident differences in the levels of this histone modification between ld and in bri1 ld mutants (Fig. 6B). In agreement with this, transcript...
expression levels of members of the PAF1 complex were not altered in bri1 ld double mutants (data not shown). We concluded from these results that bri1 probably elevates FLC expression in the ld mutant background independently from the PAF1 complex/EFS activity.

Another histone modification at the FLC locus that correlates with high FLC expression is histone acetylation (He et al., 2003; Ausin et al., 2004). This prompted us to examine whether histone H3 acetylation at the FLC locus is affected by bri1. Chromatin was immunoprecipitated with antibodies against acetylated H3 from the same tissue samples as described for histone triMeH3K4. DNA fragments of the promoter, the first exon, the first intron, and the region between the second and fourth exon of the FLC locus were amplified with PCR (Fig. 6A). We did not detect clear and reproducible enrichment in acetylated H3 in any of the tested regions in single bri1 mutants, which correlates with its lack of increased FLC expression (Fig. 6C). By contrast, in the ld mutant, we consistently detected increased H3 acetylation in all tested FLC regions, and the region around the translation initiation start, the first exon, and the 5′ region around the first intron showed the highest levels of enrichment (Fig. 6C). These regions were previously reported to be important for regulation of FLC expression and to be a target site for various chromatin modifications (Sheldon et al., 2002; He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung and Amasino, 2004). Importantly, we observed only minor differences when comparing the control Ws to any tested mutant in the region located around 1600-1900 bp upstream of the FLC coding sequence. Thus, the detected enrichment in H3 acetylation in ld mutants probably reflects increased mRNA expression of FLC. Interestingly, the double bri1 ld mutant was found to have further-enhanced enrichment in H3 acetylation (Fig. 6C). In all replicate samples tested, H3 acetylation at the FLC locus in bri1 ld mutants was consistently found to be increased compared with ld mutants in the regions around the transcription initiation start, in the first exon and in the first intron (Fig. 6C). The enhanced histone acetylation in bri1 ld, compared with ld, correlates with the elevated levels of FLC transcript found in this double mutant.

DISCUSSION

Timing of the transition to flowering is regulated by multiple endogenous and environmental factors that interact in bringing about this appropriate physiological response (reviewed in Boss et al., 2004; Komeda, 2004; Putterill et al., 2004). It seems that, despite quite intensive studies, additional factors regulating the timing of the floral transition still await discovery. In this report, we provide evidence that BRI1-dependent BR signaling is an important element in the floral-controlling network. The finding that BRs regulate FLC in the promotion of flowering was fully unexpected, and leads to new avenues to explore the floral-induction network.

We described here an enhancer screen that led to the isolation of two alleles of bri1 as modifiers of the late-flowering phenotype of the autonomous mutant ld (Fig. 1A-C). We reconstituted the late-flowering phenotype of bri1 ld double mutants isolated via double-mutant construction with a described null allele of bri1 combined with an alternative allele of ld, confirming the genetic interaction between LD and BRI1 in the control of flowering time (Fig. 1D,E). We further expanded our studies by demonstrating (using the cpd mutation) that the effect of BR deficiency on FLC expression and on flowering time in the ld mutant is comparable to the phenotypes observed for bri1 mutants. However, the flowering phenotype of cpd ld slightly differed from that of bri1 ld, depending on the counting method used to measure flowering time (Fig. 5). There are several possible explanations for this difference. One difference between cpd and bri1 is that, in the former mutant, BR production is blocked, whereas the latter mutant does produce bioactive BRs, but fails to activate the signaling pathway. In addition, in cpd mutants, the BR-synthesis pathway is blocked at the conversion step to 23-hydroxylated BRs, which probably results in the over-accumulation of brassinosteroid precursors (Szekeres et al., 1996). In the bri1 mutant, by contrast, the final products of the BR pathway – brassinolide, castasterone and tephasterol – are strongly over-accumulated (Noguchi et al., 1999). Because no physiological roles have so far been attributed to any of these precursors, we cannot exclude the possibility that these molecular differences have implications on plant fitness, growth, speed and/or minor aspects of flowering time.

Based on the severe phenotype of the bri1 ld and cpd ld double mutants, we concluded that BR activity is crucial for the correct timing of the floral transition in A. thaliana. However, both bri1 and cpd seem to function as a ‘modifier’ rather than as a strong, independent flowering-time mutant, because single bri1/cpd mutants only displayed a marginal flowering phenotype (Figs 2, 5). These weak single-mutant phenotypes and lack of reported enhancer screens of autonomous mutants are probably the reasons why bri1 and cpd have not been previously found in a range of described genetic screens for flowering-time mutants.

From the analysis of double mutants of bri1 and various known flowering-time mutants, we propose that BR signaling functions to repress the expression of FLC and that it does so independently of vernalization and of the autonomous pathway. Given that the bri1 single mutant only has a modest late-flowering phenotype, whereas the autonomous mutants or FRI plants have more-pronounced phenotypes, BRs probably have an assisting role to the autonomous pathway in the repression of FLC. In addition, bri1 does not transcriptionally regulate the autonomous pathway, because we did not detect changes in the expression of FVE, LD, FLC, FPA, FY or FLD (data not shown). The FCA member of the autonomous pathway functions as an ABA receptor in flowering-time control, and ABA regulates mRNA splicing at FCA (Razem et al., 2006). Also, BRs were reported to act antagonistically to ABA (Steber and McCourt, 2001; Friedrichsen et al., 2002), suggesting that they might also influence FCA splicing. However, we did not observe changes in the expression of the different splice forms of FCA in the bri1 single mutant or in bri1 ld compared to ld (data not shown). This indicates that BR signaling regulates FLC expression by a different mechanism other than affecting the ABA-mediated regulation of FCA splicing.

Although it has been previously reported that some photoperiod mutants increase FLC expression in certain autonomous mutant backgrounds (Rouse et al., 2002), the flowering phenotypes of the single bri1 mutant and its double mutant combinations described above are unique, and therefore make bri1 distinct from the other flowering-time mutants that have been described. Moreover, we did not observe reduced expression of CO in bri1 mutants compared to the Ws control, and presence of the bri1 mutation did not significantly alter CO levels in ld mutants (data not shown). CO is the key player in the photoperiodic response (Suarez-Lopez et al., 2001; Valverde et al., 2004). Therefore, the unchanged transcript levels of CO within bri1 mutants confirm the minor role of BR signaling in the photoperiod regulation of flowering.

The regulation of chromatin state has recently emerged as an important mechanism in the control of FLC expression (He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung and Amasino, 2004; Kim et al., 2005; Martin-Trillo et al., 2006). In particular, histone acetylation at the FLC genomic locus was found
to be correlated with actively transcribed FLC (He et al., 2003; Austin et al., 2004). Here, we demonstrate that a block in BR signaling leads to increased levels of histone H3 acetylation at the FLC locus in the ld background. It would be interesting in the future to further probe the molecular/biochemical events leading to effects of BR signaling on H3 acetylation levels at FLC chromatin.

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