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Attenuation of brassinosteroid signaling enhances *FLC* expression and delays flowering

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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, *BRI1*, *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 autonomous, and the vernalization pathways as major genetic-signaling systems promoting flowering in *A. thaliana* (Koornneef et al., 1991; Wilson et al., 1992; Koornneef et al., 1998). Photoperiod-pathway mutants [e.g. *constans* (*co*) and *gigantea* (*gi*)] exhibit a strong late-flowering phenotype under long-day growth conditions (Koornneef et al., 1991; Putterill et al., 1995; 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).

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 *FPA* (Koornneef et al., 1991; Lee et al., 1994; Chou and Yang, 1998; Schomburg et al., 2001; Lim et al., 2004; Mockler et al., 2004). This pathway acts to negatively regulate the expression of the potent floral repressor *FLOWERING LOCUS C* (*FLC*), because autonomous mutants were found to have elevated levels of *FLC* transcript and their late-flowering phenotype is suppressed by a loss-of-function mutation of *flc* (Michaels and Amasino, 1999; Sheldon et al., 2000; Michaels and Amasino, 2001).

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 *A. 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 IN SHORT DAYS 1 [ESD1; also known as ACTIN-RELATED PROTEIN6 (ARP6) and ATARP6 – TAIR] was recently demonstrated to be essential for H3 acetylation and triMeH3K4 in the *FLC* chromatin (Martin-Trillo et al., 2006).

Brassinosteroids (BRs) are steroid hormones known to control various aspects of plant development, including skotomorphogenesis, photomorphogenesis and xylem formation, as well as cell division and elongation (reviewed in Nemhauser and Chory, 2004; Asami et al., 2005). BRs are recognized by a plasma membrane-localized leucine-rich-repeat receptor-like kinase (LRR-RLK), BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li and Chory, 1997; Friedrichsen et al., 2000; He et al., 2000; Wang et al., 2001; Kinoshita et al., 2005). BRI1 consists of an extracellular region containing 24 LRRs interrupted by the so-called island domain, followed by a transmembrane domain and a cytoplasmic serine/threonine kinase domain (Li and Chory, 1997; Vert et al., 2005). BRs bind to the island domain and the neighboring LRR, which initiates a BR signal transduction cascade via the kinase activity of BRI1 (Wang et al., 2001; Kinoshita et al., 2005; Wang et al., 2005). BRI1 functions as the major receptor for BRs, because the phenotype of strong loss-of-function *bri1* mutants resembles a severe BR deficiency (Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory, 1997). Both BR-deficient mutants and *bri1* mutants were reported to be marginally late flowering, whereas the *bas1 sob7* double mutant, impaired in metabolizing BRs to their inactive forms, exhibited modest early flowering (Chory et al., 1991; Li and Chory, 1997; Azpiroz et al., 1998; Turk et al., 2005). Thus, it seems that BRs and BRI1 play a promoting role in the floral transition, but, thus far, no detailed study on this subject has been reported.

Here, we provide evidence that BRs regulate the timing of the floral transition by regulating *FLC* expression. We identified two alleles of *bri1* as strong enhancers of the autonomous mutant *luminidependens* (*ld*). We further show that *bri1* phenotypically enhances *FRI* and the autonomous mutant *fca* in a similar manner, leading to an increase in *FLC* transcript levels. The extremely late-flowering phenotype of *bri1 ld*, *bri1 fca* and *bri1 FRI* double mutants can be suppressed by a prolonged exposure to cold (vernalization). Moreover, specific reduction of *FLC* by RNA interference (RNAi) profoundly accelerated flowering of *bri1 ld* mutants, confirming that high *FLC* mRNA abundance is the major cause of the flowering behavior of this double mutant. In addition, the BR-deficient mutant *cpd* enhanced the late flowering of *ld* and increased *FLC* transcript levels, resembling the phenotypes we found for *bri1* mutants. This indicates to us that the effects of *bri1* 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^{SF2} is a Ws line with the *FRI* allele from San Feliu-2 (SF2) (Lee et al., 1993). *gi-11* was provided by J. Putterill (Fowler et al., 1999) and *cpd–3939* was a gift from F. Tax (Noguchi et al., 2000). The *ga1-3* mutant, provided by S. Bednarek (University of Wisconsin-Madison, Madison, WI), originally in the L*er* 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 *ga1* 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 sulphonate, 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-*att*B1-CCCGAGAAAAGGAAAAAAAAAAATA-3′ and 5′-GGGG-*att*B2-CGGCTTCTCTCCGAGAGGG-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 pJawohl8-RNAi (provided by B. Ülker and Dr I. Somssich, 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.

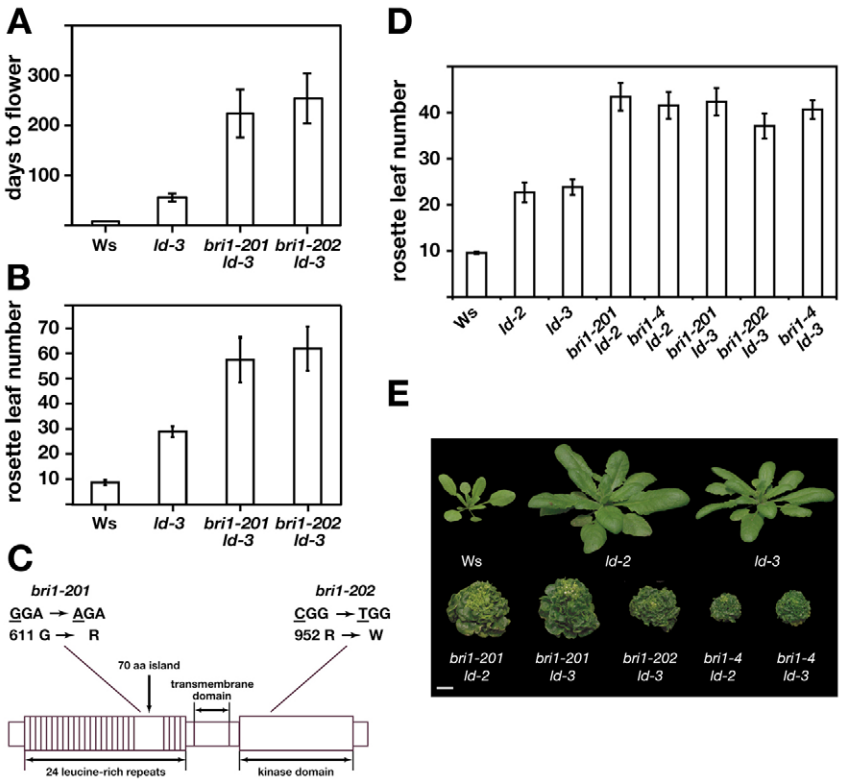

Fig. 1. *bri1* is an enhancer of the autonomous mutant *ld*. (**A**,**B**) Flowering time under long days (16 hours light/8 hours darkness) of the wild-type control (Ws), the *ld-3* single mutant and the two double *bri1 ld* mutants isolated in the enhancer screen of *ld–3*, measured as (A) days to flower or (B) rosette leaf number at bolting. (**C**) Schematic representation of the effect of the *bri1-201* and *bri1-202* mutations at the gene and protein level. (**D**) Flowering time of various *bri1 ld* double-mutant combinations under long days. A representative experiment is shown. Flowering time was measured as the total number of rosette leaves formed when the bolt was approximately 1 cm. The leaf number values are averages from 10-18 plants per genotype. Error bars represent s.e.m. (**E**) Photographs at bolting of *bri1 ld* double mutants compared with Ws and the single mutants *ld-2* and *ld-3*. Scale bar: 1 cm.

Analysis of *FLC* mRNA abundance

Tissue was harvested from the aerial parts of plants 9 hours after dawn. Total RNA was isolated with the Plant RNeasy kit (Qiagen, Hilden, Germany). RNA (7.5-15 μg) was separated on a 1.5% agarose denaturing formaldehyde gel and transferred to the Hybond NX membrane (Amersham Biosciences, Freiburg, Germany). The *FLC* probe was 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′, 5′-GGATAGCATGTGGAAGTGCATACC-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 with the RNeasy Plant Mini 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 H_2O to a final volume of 80-100 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 *UBQUITIN 10* (*UBQ10*, also known as *POLYUBIQUITIN 10* – TAIR) with the following primers: 5′-TAAAAACTTTCTCTCAATTCTCTCT-3′ and 5′-TTGTCGATGGTGTCGGAGCTT-3′. PCR products were separated on ~2.5% agarose gels. The DNA was stained with ethidium bromide and visualized using a PhosphorImager (Molecular Dynamics, USA).

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′-GCACATGCCCTACCCATGAC-3′, 5′-CCCAAATCTTTGGCTACCATCG-3′, and 5′-TGTGTTACCATTCAAACGGTATAATCT-3′, 5′-TCCACACATATGGCAATAGCTCAA-3′, respectively. Primers III to IX correspond to primers A to G, as described (Bastow et al., 2004). A primer pair specific for *UBQ10* (5′-TCGTTCGATCCCAATTTCGT-3′ and 5′-CAAATTCGATCGCACAAACT-3′) was used to amplify a fragment as an internal control for ChIP. 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 *luminidependens* (*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 *BRI1* 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 was comparable to the observed phenotypes in the

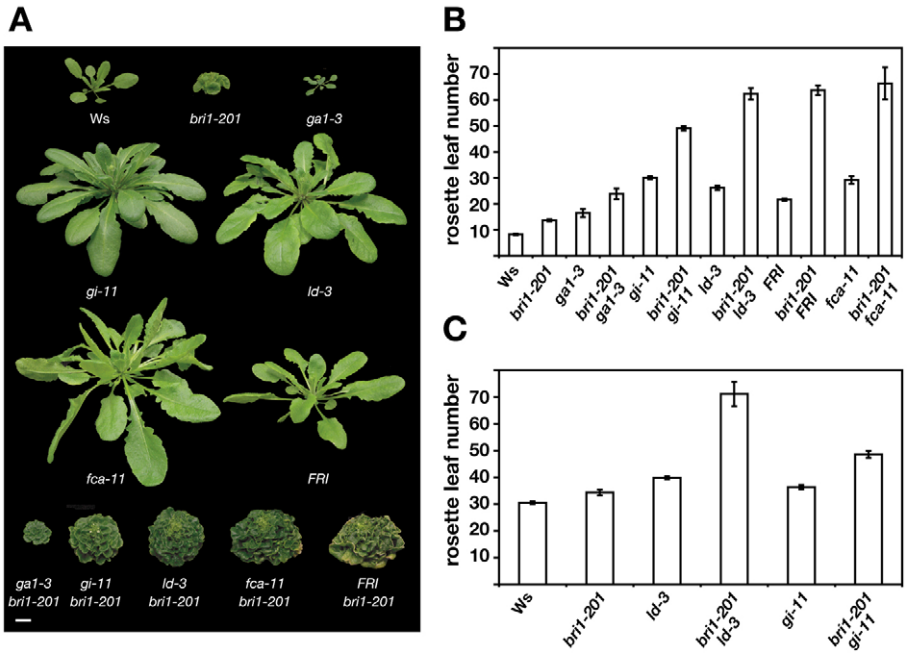

Fig. 2. *bri1* is a strong enhancer of *FRI* and of autonomous mutants. (**A**) Photographs at bolting of double mutants between the *bri1-201* mutant and different flowering-time mutants: autonomous (*ld-3*, *fca-11*), photoperiod (*gi-11*), gibberellin (*ga1-3*), and dominant *FRI* in the Ws background. Each single mutant is also shown. Plants were grown under long days. (**B**) Flowering time under long days (16 hours light/ 8 hours darkness). (**C**) Flowering time of the *bri1-201*, *ld-3* and *gi-11* single mutants and the respective *bri1* double mutants under short days (10 hours light/14 hours darkness). Representative experiments are presented. Flowering time was measured as in Fig. 1. Scale bar: 1 cm.

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

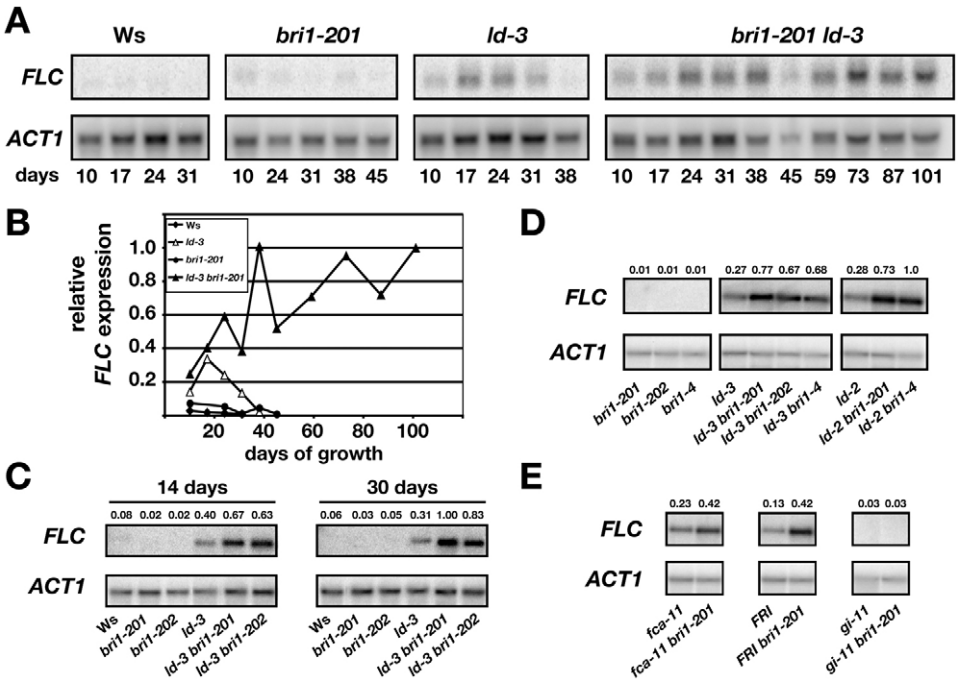

Fig. 3. *bri1* mutation leads to an elevation of *FLC* mRNA levels in *FRI* and in autonomous mutants. Absolute (**A**) and relative (**B**) *FLC* expression in *bri1-201 ld-3* double mutants, compared to the respective single mutants, under long-day photoperiods. Samples were taken at the time indicated (number of days of growth), until the flower buds were visible. (**C**) *FLC* expression in *bri1-201 ld-3* and *bri1-202 ld-3* double mutants and in the respective single mutants after 14 and 30 days of growth under short days. (**D**) *FLC* mRNA levels in 30-day-old *bri1 ld* double-mutant combinations, and (**E**) in the double-mutant *bri1 fca*, *bri1 FRI* and *bri1 gi* lines under long days. *FLC* mRNA abundance was monitored by RNA-blot analysis. (A-E) The *ACTIN 1* (*ACT1*) gene was used as a loading control. *FLC* levels were quantified, values were normalized to the levels of *ACTIN 1*, and the highest value in each separate experiment was set to be 1.

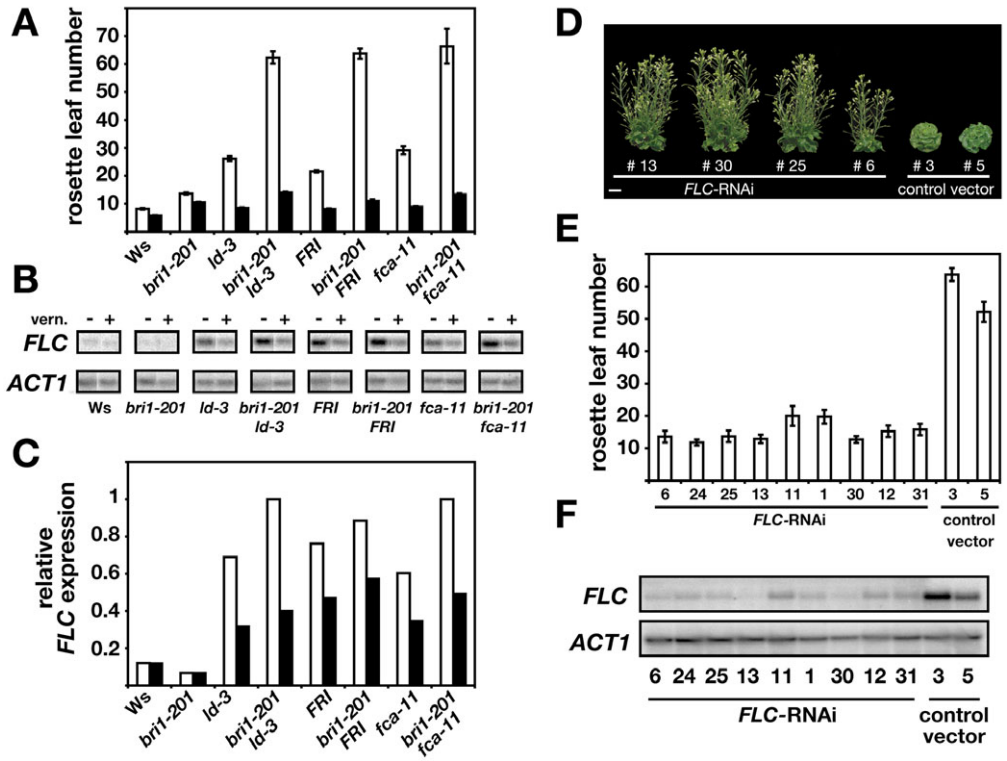

Fig. 4. Reduction of *FLC* expression in *bri1 ld*, *bri1 fca* and *bri1 FRI* double mutants efficiently accelerates flowering. (**A**) The effect of vernalization treatment on flowering time in the following mutants: *bri1-201*, *ld–3*, *fca-11*, *FRI* in the Ws background, *bri1-201 ld-3*, *bri1-201 fca-11* and *bri1-201 FRI*. Germinated seedlings were vernalized for 6 weeks under short days (8 hours light/16 hours darkness) at 4°C, after which they were grown under the same long-day conditions as control non-vernalized plants. White bars, non-vernalized controls; black bars, vernalization treatments. Absolute (**B**) and relative (**C**) *FLC* mRNA levels before (–; white bars) and after (+; black bars) vernalization. (**D**) Flowering phenotypes of four independent *FLC*-RNAi transgenic lines in the *bri1-201 ld-3* double-mutant background, compared to two independent lines of *bri1-201 ld-3* double mutants transformed with a control vector. 7-week-old plants are shown. (**E**) Flowering-time analysis of *FLC*-RNAi *bri1 ld* mutants grown under a long-day photoperiod. A representative result is shown. (**F**) RNA-blot analysis of *FLC* mRNA levels in 20-day-old *FLC*-RNAi *bri1 ld* mutants compared to the control *bri1 ld* lines. The *ACT1* gene was used as a loading control.

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).

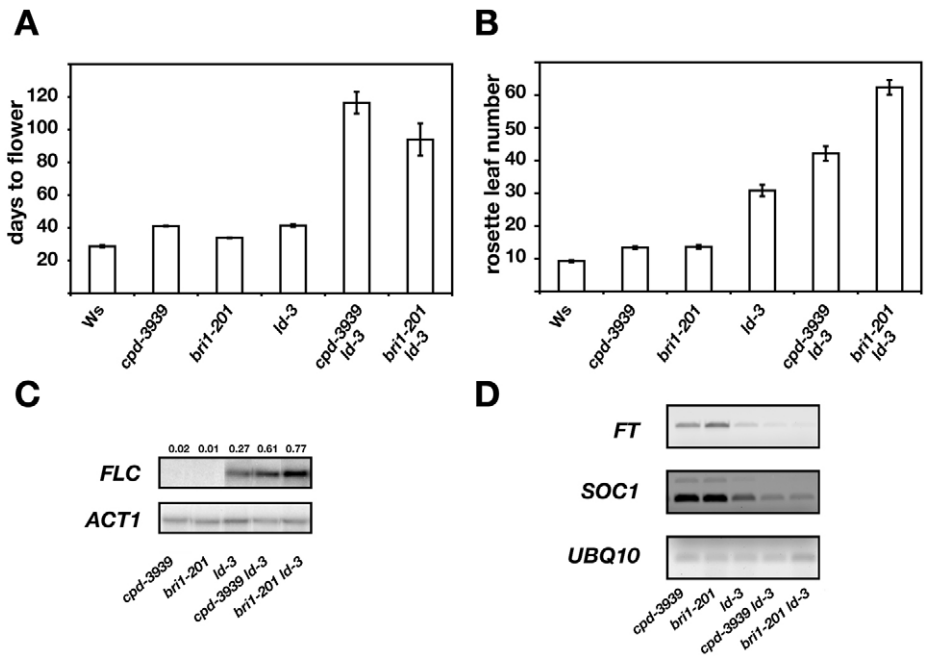

Fig. 5. Combining the BR-deficient mutant *cpd* with *ld* delays flowering and enhances *FLC* expression. (**A**,**B**) Flowering time, under long days, of *cpd-3939 ld-3* compared to *bri1-201 ld-3* mutants and to the respective single mutants, measured as either days to bolt (A) or rosette leaf number at bolting (B). A representative result is shown. (**C**,**D**) *FLC* expression (C) and the abundance of the floral pathway integrators *FT* and *SOC1* (D) in the 30-day-old plants described in A. *FLC* mRNA abundance was monitored as in Fig. 1. *FT* and *SOC1* mRNA levels were measured by reverse transcriptase (RT)-PCR and *UBQ10* was used as a control.

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 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 BRI1 as an important modulator of flowering time, we wondered whether the observed effects reflect the role of BRI1 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 BRI1-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

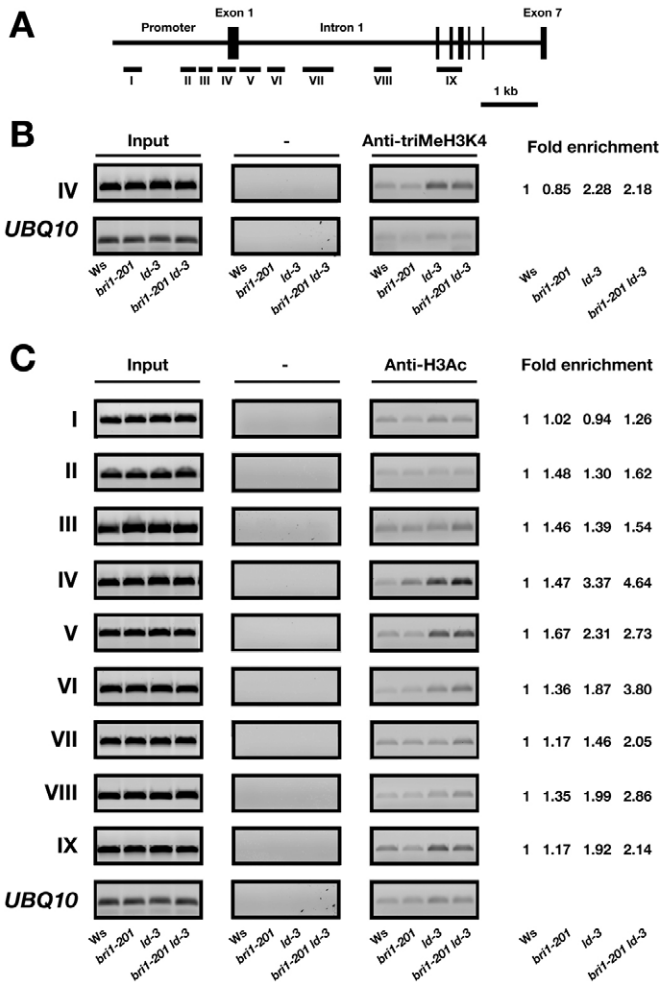

Fig. 6. Histone acetylation at the *FLC* locus is increased in *bri1 ld* mutants. (**A**) Regions of the *FLC* locus examined by ChIP. Coding regions are indicated with vertical lines, and the nine fragments amplified with PCR are depicted and numbered from I to IX. (**B**) ChIP with antibodies against trimethylated histone 3 at lysine 4 (triMeH3K4). 21-day-old Ws, *bri1-201*, *ld-3* and *bri1-201 ld-3* mutants grown under long days were analyzed, and region IV was examined. (**C**) ChIP with antibodies against acetylated histone 3 (H3Ac). Samples are as described in B. (B,C) Input, denotes DNA amplified from each chromatin sample before immunoprecipitation. –, denotes samples immunoprecipitated with antibodies against anti-rat-IgG (negative control). Anti-H3Ac refers to fragments amplified from chromatin samples immunoprecipitated with antibodies against H3Ac, whereas Anti-triMeH3K4 refers to samples immunoprecipitated with antibodies against trimethylated H3K4. *UBQ10* served as an internal ChIP control. Representative ChIPs are illustrated. Fold enrichment calculated for a biological replicate is the mean of averaged enrichment ratios from two independent immunoprecipitations. Fold enrichment in Ws was arbitrarily set as 1.

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 typhasterol – 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*, *FLK*, *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; Ausin 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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