Genetic analyses of brassinosteroid control of flowering time in *Arabidopsis thaliana*

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Abbreviations

aa	amino acid
ABA	abscisic acid
bp	base pair
BL	brassinolide
BRs	brassinosteroids
BSA	Bovine serum albumin
°C	degree Celsius
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
dH_2O	deionized water
DNA	deoxyribonucleic acid
dsRNAi	doublestranded-RNA-interference
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
GAs	gibberellins
GUS	□-glucuronidase
h	hour
l	liter
LRR	leucine-rich repeats
m	mili
M	molar
min	minute
MES	2-(N-morpholino)ethane sulfonic acid
MOPS	3-(N-morpholino)propane sulfonic acid
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SAM	Shoot apical meristem
SE	standard error
SDS	sodium dodecyl sulphate
UTR	untranslated region
(v/v)	volume per volume
(w/v)	weight per volue

1. Introduction

1.1. Transition to flowering

As sessile organisms, plants had to evolve many strategies that would allow them to survive and reproduce under continuously changing environmental conditions. Two plant-specific features that enable them to effectively compete for essential resources are an indeterminate growth habit and the extreme plasticity in their development. The first strategy provides plants with the capacity for "unlimited" growth, due to the continuous activity of meristems. The plasticity of growth, in turn, is the ability to adjust developmental programs in response to variations in the environment. The interplay between the activities of the genetic complement of a plant and the influence of diverse environmental factors brings about the morphological and physiological responses that regulate plant growth.

During postembryonic development, plants undergo three defined temporal phases: a juvenile vegetative phase, an adult vegetative phase, and a reproductive phase. The transition from vegetative to reproductive development is the most dramatic phase change. Timing this transition, so that it occurs under the most advantageous conditions for pollination and seed production is absolutely essential for the maximization of plant's reproductive success (Chuck and Hake, 2005; Poethig, 2003).

The transition from vegetative to reproductive growth occurs in the shoot apical meristem (SAM) (Fig. 1.1). During the vegetative phase, the SAM gives rise to lateral meristems that develop into leaves. Various environmental and endogenous signals that promote flowering induce an array of biochemical and cellular changes that alter the developmental fate of the SAM, such that it starts initiating floral primordia. This ultimately leads to the development of the floral organs: sepals, petals, anthers, and carpels. Together these structures represent the flower (Koornneef *et al.,* 1998)

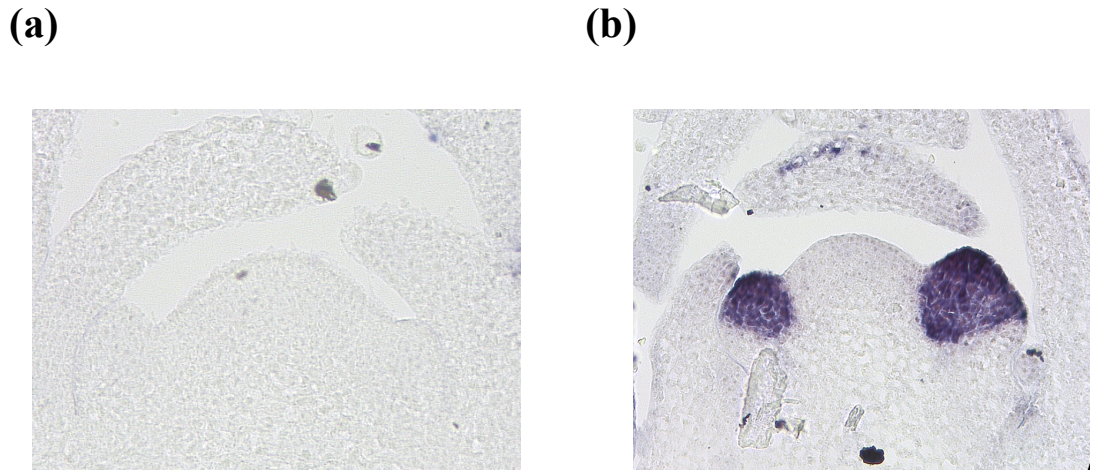

Figure 1.1. The shoot apical mersitem (SAM) is the site of transition to flowering. The vegetative meristem (a) and the meristem upon the transition to flowering (b). Pictures show *in situ* hybridization with probe specific for a marker that denotes the floral transition has occured. (a) Plants were grown for 14 days under non-inductive conditions for flowering (short days) (b) Plants were grown as in (a) and transferred to floral-inductive conditions (long days) and grown for additional 72 h. Purple staining indicates floral-specific marker *AP1* that marks the site where flowers are initiated. Photos kindly provided by I. Searle and C. Vincent.

1.2. Arabidopsis as a model to study floral transition

The introduction of *Arabidopsis thaliana*, a small weed from the family *Brassicaceae*, as a model system, has greatly facilitated studying genetic and molecular basis of various physiological processes regulating plant development. As early as 1943, Friedrich Laibach described the advantages of using Arabidopsis for genetic studies and proposed to its use as a model system (Laibach, 1943). The features that make Arabidopsis a suitable system for genetic studies include a diploid genome, the small size of the plant, a short generation time, self-fertilization that enables easy maintaining the mutation of interest, high seed yield, and that it is not difficult to grow in the very well defined environment (Laibach, 1943). Later on, it was recognized that Arabidopsis has one of the smallest known plant genome, with fewer repetitive sequences, and as well, it can be easily transformed, which makes it an excellent model for genomic and molecular studies. Arabidopsis turned out to be particularly useful for forward-genetics in which mutagenized populations are screened for phenotypes of interests. Hence, the forward-genetic approach leads

towards isolating genes involved in the control of the chosen biological process. Thanks to the described features, Arabidopsis became a model plant system and has been successfully applied for the analyses of the control of flowering time (Page and Grossniklaus, 2002).

George Redei was one of the first scientists that recognized the power of Arabidopsis as a genetic model. In 1961, he reported identification of "supervital" mutants, which he named *luminidependens* (*ld*), *constans* (*co*), and *gigantea* (*gi*) (Redei, 1963). These mutants were the first flowering-time mutants described in Arabidopsis. Much later, numerous screens were performed aiming to find early/late flowering plants in the varying light regime, mutants with altered response to known inductive treatments, such as prolonged exposure to cold, *etc*. These screens identified thus floral inducers and repressors. Based on these studies, genetic pathways that regulate floral transition in Arabidopsis were described. Initially, four main genetic pathways were established derived from the late-flowering phenotypes of mutants specific for respective pathways (Redei, 1962, Koornneef *et al.,* 1991). These pathways define the role of the inductive photoperiods, a class of phytohormones (the gibberellins [GAs]), prolonged exposure to cold, and autonomous factors in the control of flowering time. Further analyses increased the complexity of our understanding of the floral promotion by including influence of light quality, ambient temperature, and other factors into the model (Mouradov *et al.,* 2002, Henderson *et al*., 2004; Boss *et al.,* 2004; Putterill *et al.,* 2004) (Fig. 2). Known floral-regulating pathways converge on a small number of genes, called floral-pathways integrators, Up to now, three genes that have been shown to perform this function: *FLOWERING LOCUS T* (*FT*), *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and *LEAFY* (*LFY*). (Samach *et al.,* 2000; Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Blazquez *et al.,* 1997). Floral-pathways integrators activate floral-meristem identity genes, and that triggers the transition from vegetative to reproductive phase (Henderson *et al*., 2004).

1.2.1. Photoperiod pathway

One of the most potent environmental cues that regulates flowering time is based on the duration of the light period within a 24h day, also termed photoperiod. Arabidopsis is a facultative long-day plant, it flowers earlier under long - day conditions, while short days delay the time of floral transition (Searle and Coupland, 2004). In 1936, Erwin Bünnig proposed that measurements of photoperiods depended on an endogenous diurnal rhythm. According to his model, an autonomous mechanism (termed the circadian clock) generates rhythms with an approximately 24-h period that can be divided into phases of different sensitivity to light. Growing plants in photoperiods that expose them to light during the light-sensitive phase triggers or represses the flowering response (Bünnig, 1936). This concept was further developed into the external-coincidence model, in which the temporal interaction between a circadian rhythm and light (acting as an external stimulus) defines the basis for day-length measurement (Thomas and Vince Prue, 1997). Recent molecular studies support this model for the photoperiodic control of flowering in Arabidopsis (Yanovsky and Kay, 2003; Hayama and Coupland, 2004).

The circadian system can be divided into three parts: the central oscillator, the input, and the output pathways. The central oscillator is the core of the system and it generates the 24-h time keeping mechanism. Input pathways such as light or temperature signals entrain the central clock to synchronize to the daily cycles. The central oscillator regulates output pathways, which in turn regulate a range of developmental processes. Flowering time is believed to be regulated by one or more of such output pathways (Searle and Coupland, 2004; Hayama and Coupland, 2004).

Genetic studies using Arabidopsis revealed that several mutants impaired in different parts of the circadian system were affected in the photoperiodic control of flowering time. Some of the identified genes are implicated in the light input to the clock: photoreceptors (phytochromes and cryptochromes), which perceive light, and *EARLY FLOWERING 3* (*ELF3*) and *ZEITLUPE* (*ZTL*), which mediate between photoreceptors and the clock. Mutations that disrupt central oscillator function include *late elongated hypocotyl (lhy), circadian clock associated 1 (cca1)* and *timing of chlorophyll a/b binding protein1 (toc1)*. These mutants have reduced photoperiod

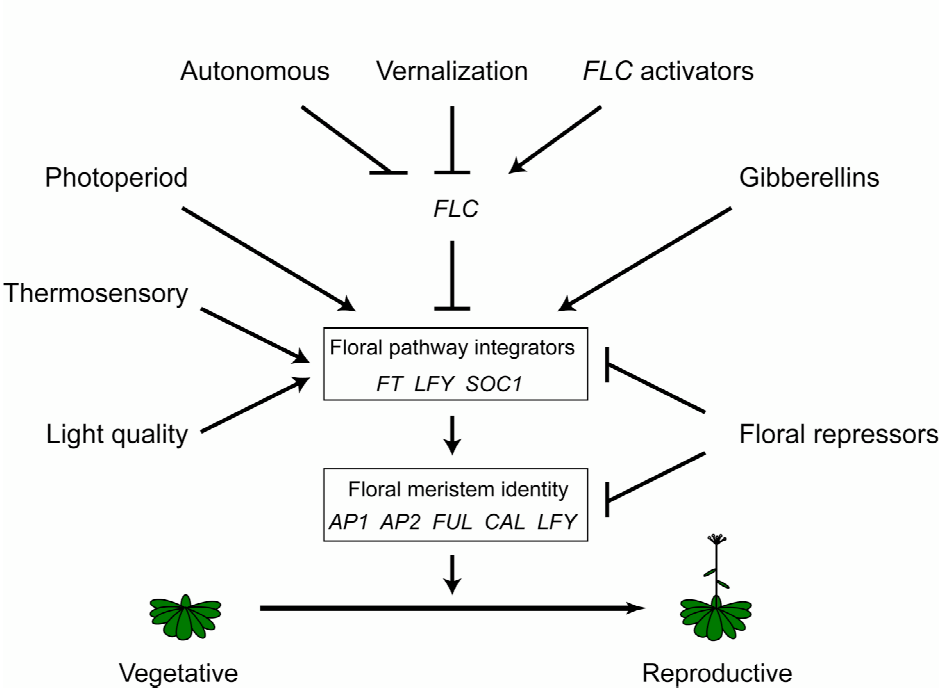

Figure 1.2. A simplified model of pathways controlling flowering time in Arabidopsis. The photoperiod pathway is predominant under long days, while under short-day conditions, flowering is promoted by gibberellins. The thermosensory, the light quality, and the vernalization pathways modulate flowering time in response to environmental cues, such as ambient temperature, the spectral composition of light, and prolonged exposure to low temperatures, respectively. The autonomous pathway functions in parallel to the vernalization pathway to repress the expression of the potent floral repressor, *FLC*. Flowering is delayed by a heterogenous group of floral repressors that either directly repress floral pathway integrators and/or floral meristem identity genes or activate the expression of the strong floral repressor, *FLC*. Distinct genetic pathways ultimately converge on a small number of genes called floral integrators: *FT, SOC1* and *LFY*. The floral integrators upregulate the expression of the floral meristem-identity genes *AP1*, *AP2*, *FUL*, *CAL*, and *LFY*. The induction of these genes results in a meristem transition such that lateral organs produced are flowers instead of leaves. Adapted from Henderson *et al.* (2004).

response and are early flowering under short days. *EARLY FLOWERING 4* (*ELF4*) is a candidate gene to act in the circadian clock, whose loss-of-function leads to a similar early flowering phenotype under short days (Searle and Coupland, 2004).

Mutants that flower late under long days, but have a nearly wild-type-flowering phenotype under short days, define a group of genes regulated by the circadian clock (Koornneef *et al.*, 1991, Searle and Coupland, 2004). These include *GIGANTEA* (*GI*), *FLAVIN-BINDING, KELCH REPEAT, F-BOX* (*FKF1*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*). *GI* and *FKF1* control

transcription of *CO*, which in turn activates expression of *FT* (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Borner *et al*., 2000; Onouchi et al., 2000; Samach et al., 2000). The latter is a flowering-time gene that is also regulated by other floral-promoting pathways. Recent studies show that the interaction between circadian rhythms and light signaling may happen at the level of transcriptional and posttranscriptional regulation of CO. This interaction defines a molecular basis for external coincidence model of photoperiodic control of flowering time, confirming Bünnig hypothesis. In this model, *CO* mRNA levels define the light-sensitive phase, and CO protein stability is differentially regulated by light throughout the day. Under short days, CO expression is the highest in the night and the result is that CO protein does not accumulate. Under long days, in turn, *CO* mRNA coincides partially with light and CO protein is stabilized at the end of the day. The accumulation of CO, at the end of a long day, directly induces expression of *FT* to strongly promote flowering under such an inductive photoperiod (Suarez-Lopez *et al.,* 2001; Roden *et al*., 2002; Yanovsky and Kay, 2002; Imaizumi *et al*., 2003; Valverde *et al*., 2004).

1.2.2. Light quality

The spectral composition of light constitutes a distinct visual cue that regulates flowering via the light-quality pathway. The effect of the light quality on flowering time differs from light input in the photoperiod pathway, because it provides information about the local environment in which the plant grows, rather than global information about the seasonal changes. A well described example of the light-quality effect on plant physiology is the shade-avoidance response. In light that reaches plants growing at the base of the canopy, the red to far-red ratio is lower due to the reflection and chlorophyll absorption of red light. As a result plants must compete for the best growth conditions and far-red light serves as a signal of a "crowded environment". This signal induces many physiological responses, of which early flowering is one example (Simpson and Dean, 2002, Boss *et al.,* 2004).

The importance of light quality in the control of flowering time in Arabidopsis has been confirmed by molecular-genetic studies. In general, it is believed that red light delays flowering through the red-light receptor phytochrome B (phyB) and to a

lesser extent, through phyD and phyE, while far-red and blue light promote flowering through PHYA and CRYPTOCHROMES CRY1 and 2, respectively. This notion is supported by the phenotype of photoreceptor mutants, which exhibit altered flowering time. In particular the *phyB* flowers early suggesting that phyB is a repressor of flowering (Halliday et al., 1993, Aukerman et al., 1997; Devlin et al., 1998, 1999). The *phyA* mutant is slightly late flowering under long days, and strongly delayed when the light is far-red enriched at the end of the light period, or when the night is interrupted by a short period of light (Reed et al., 1994). This indicates that phyA promotes flowering in these conditions. Finally, *cry2* flowers late under long days, demonstrating that CRY2 functions to promote flowering (Lin, 2000). The elements acting downstream from photoreceptors in this pathway await isolation and characterization.

1.2.3. Ambient-temperature (thermosensory) pathway

Ambient temperature represents another environmental signal that regulates floral timing. Classical physiological studies showed that growth temperature affected timing of the floral transition, by inducing or inhibiting flowering, and high ambient temperature could substitute for day length or vernalization effect, depending on the species studied (Bernier et al., 1993). However, the molecular and genetic basis underlying these physiological effects is poorly understood. Emerging evidence indicates that two autonomous pathway (described below) genes *FVE* and *FCA* are likely to control the ambient-temperature pathway (Blazquez *et al.,* 2003). In addition, various photoreceptors: phyB, phyD, phyE, phyA, CRY1 and CRY2 have been shown to have differential activity at 16°C and 22°C, suggesting the cross-talk between the light quality and the ambient temperature pathways (Blazquez *et al.,* 2003, Halliday *et al*., 2003). Further studies should reveal other components of this pathway and answer whether the thermosensory pathway interacts with other known flowering-time pathways.

1.2.4. Gibberellin pathway

The role of gibberellins in floral promotion was first demonstrated in 1957 by Langridge, who observed that exogenous application of gibberellins accelerated flowering (Langridge, 1957). These physiological observations were later confirmed by genetic studies showing that mutants blocked in GA-biosynthesis (*gibberellin deficient1 [ga1])* and signaling (*gibberellin insensitive [gai])* are late-flowering (Wilson et al., 1992). Their phenotype is mild under long days and very severe under short-day conditions. Furthermore, mutants with enhanced GA-signalling, such as *spindly* (*spy*) and plants overexpressing *FLOWERING PROMOTIVE FACTOR1* (*FPF1*), which is believed to be involved in GA-signal transduction, flower early (Jacobsen and Olszewski, 1993, Kania *et al.,* 1997). The increase in the level of endogenous GAs caused by overexpression of the GA20 oxidase *GA5*, leads to a similar early flowering-time phenotype, particularly in short days (Huang *et al.,* 1998; Coles *et al.,* 1998). Double-mutant analyses revealed that the GA pathway is distinctive from the photoperiod and the autonomous pathways and that its activity is most important during growth under a non-inductive photoperiod. Nevertheless, a loss of function allele of the autonomous gene, *FPA* has been identified in the screen for components of GA signaling, indicating that there is a certain level of crosstalk between the gibberellin pathway and the autonomous pathway (Schomburg et al., 2001). Additionally, in some species, GA treatment can substitute for vernalization (prolonged exposure to low temperatures) and it has been suggested that vernalization acts via the GA pathway (Zeevart, 1983). However, this is not the case in Arabidopsis, where the GA and the vernalization pathways function independently. Interestingly, the GA pathway activates the floral integrators *SOC1* and *LFY* at the promoter level (Blazquez *et al.,* 1998; Moon et al., 2003).

1.2.5. Other phytohormones

The role of other hormones in the control of flowering time has been suggested based on either physiological studies or the analyses of mutants impaired in biosynthesis or signalling of the hormone of interest (Boss et al., 2004). Only for

gibberellins has the role in regulating the transition to flowering been well-documented at both the physiological and molecular-genetic level (described in detail above).

The importance of ABA in regulating the floral transition has been initially proposed based on the early-flowering phenotype of the Arabidopsis ABA-deficient and –insensitive mutant *abi3,* indicating that ABA inhibits flowering (Martinez-Zapater *et al.,* 1994). In a very recent study, ABA has been shown to influence floral transition by direct binding to RNA-binding protein FCA, which is a described member of the autonomous pathway (described below) that promotes flowering through downregulation of the potent floral repressor *FLOWERING LOCUS C (FLC*). Binding of ABA to FCA leads to the disruption of FCA-FY complex, which, through an unknown mechanism, negatively regulates expression of *FLC*. Thus, ABA application leads to accumulation of *FLC* transcript that results in a delay in flowering. This indicates that ABA, at least in part, regulates flowering by affecting the activity of some elements of the autonomous pathway (Razem *et al*., 2006). Achard *et al*. (2006) have independently demonstrated the inhibiting role of ABA on flowering time in the study where they also analyzed the effect of ethylene and salt on various aspect of plant growth. They showed that salt, ABA, and ethylene extend the vegetative phase through a common molecular mechanism. However, according to their results salt (which activates ABA and ethylene signalling) delays flowering mostly through the repression of the expression of *LFY*, whereas the level of *FLC* transcript is only slightly altered. Interestingly, even though the ABA and the ethylene pathways are involved in plant responses to diverse abiotic and biotic cues, their role in the control of flowering seems not to require the activity of the known salt-induced downstream targets (Achard *et al.,* 2006). In contrast, ethylene-signaling mutants exhibit a late-flowering phenotype, suggesting that ethylene functions to promote flowering (Guzman and Ecker, 1990). Hence, the function of ethylene in floral promotion needs to be studied in more detail.

Other phytohormones have also been implicated in the floral transition. Mutants with decreased levels of salicylic acid (SA), including transgenic nahG plants, and mutants defective in SA biosynthesis the *eds5/sid1* and *sid2* mutants, defective in SA biosynthesis, are delayed in flowering, particularly under short days.

It has been suggested that the regulation of flowering by SA may involve both the autonomous and the photoperiod pathways (Martinez *et al*., 2004). The function of another group of hormones, cytokinins has been established by physiological studies, but genetic data to support their role in floral transition are lacking. Brassinosteroids (BRs), a class of plant steroid hormones, have also been postulated to promote flowering, based on the late-flowering phenotype of the BR-deficient mutants, *det2* and *dwf4* (Chory et al., 1991; Azpiroz et al., 1998). Moreover the *bas1 sob7* double mutant, that is impaired in metabolizing BRs to their inactive forms, flowers slightly earlier, supporting the promoting role of BRs in floral transition (Turk *et al.,* 2005).

Despite a large amount of data describing the physiological roles of hormones and knowledge about molecular mechanisms of hormones action, their role in the control of flowering has just begun to be investigated in detail and awaits thorough characterization.

1.2.6. Nitric oxide

Recent studies revealed that nitric oxide (NO) represses flowering in Arabidopsis. NO is a signaling molecule, whose production is induced by various abiotic and biotic stresses such as drought, salt stress, and pathogen infection. The NO-deficient mutant *nos1* flowers early, while plants overproducing NO due to disruption of the chloroplast phosphoenolpyruvate/phosphate translocator gene *NOX1*, are late flowering. Also, exogenous application of sodium nitroprusside that serves as a NO-donor, results in delayed flowering. Further molecular studies showed that NO represses flowering by repressing the photoperiod pathway and upregulating the expression of floral repressor *FLC* (He *et al*., 2004). However, the biological relevance of NO-mediated inhibition of flowering still remains to be investigated.

1.2.7. Floral repressors

Numerous genetic screens have resulted in the identification of early-flowering mutants.These are, among others, *terminal flower1* (*tfl1*), *short vegetative phase* (*svp*), *target of eat1/2* (*toe1/2*), *schnarchzapfen* (*snz*), *schlafmutze* (*smz*),

embryonic flower 1 (*emf1*), *embryonic flower2* (*emf2*), and *terminal flower2* (*tfl2*), *fertilization-independent endosperm* (*fie*), *curly leaf* (*clf*), *early bolting in short days* (*ebs*), *early in short days4* (*esd4*), *early flowering3* (*elf3*), and *elf4* (Sung *et al.,* 2003). Hence, the wild-type alleles of these genes function genetically to promote the vegetative growth or to repress the reproductive growth. Currently it appears that flowering is a "default" developmental program, which is repressed to maintain a vegetative growth (Sung *et al.,* 2003). This repression is exerted by actively repressing genes that are required for flower development. The floral repressors can be divided into two classes: specific repressors of floral pathway integrators, *eg*. *FLC* (described in detail below), *SVP*, *TFL1*, and genes that have pleiotropic effects on plant development. The second class include *FIE, CLF, EMF1, EMF2, TFL2,* and *EBS*, which are putative regulators of chromatin state (Sung *et al.,* 2003).

A large group of floral repressors are *AP2*-like genes, *SMZ, SNZ, TOE1* and *TOE2*, which are downregulated upon photoperiodic floral induction (Puterill *et al.,* 2004). Interestingly, *TOE1* and *TOE2* were the first two flowering-time genes shown to be regulated by microRNA (Aukerman, Sahai, 2003; Chen, 2004) .

1.2.8. Vernalization pathway

Vernalization promotes flowering in response to prolonged exposure to low temperatures. The vernalization response evolved to distinguish between long periods of cold that occurs during winter and the temperature fluctuations that might occur in Fall. Vernalization is highly quantitative; the longer the cold exposure, the stronger is the acceleration of flowering. Vernalization establishes a cellular "memory" that is stable through mitosis. Therefore, the period of cold treatment and the onset of flowering can be temporally separated. Vernalization provides the competence to respond to floral-promoting signals, such as increasing day length in spring (Amasino, 2005).

In Arabidopsis, two types of accessions that differ in their response to vernalization have been identified: summer and winter annuals. Summer annuals (the so-called rapid cyclers) germinate in Spring or early Summer and flower the same year. Most commonly used laboratory strains (*e.g.* Col-0, Ws-2, L*er*) are rapid

cyclers. Winter annuals (biennials) in turn, germinate in the Fall, and grow vegetatively through Winter to flower in the following Spring (Amasino, 2005). By crossing a summer to a winter-annual of Arabidopsis, in the 1950s, Napp-Zinn identified a single dominant locus termed *FRIGIDA* (*FRI*) as a major determinant of a vernalization requirement (Napp-Zinn, 1987). More recently, studies of natural variation in Arabidopsis led to identification of *FLC* as another major determinant, critical in establishing the winter-annual habit. The presence in Arabidopsis of dominant variants of both loci, thus confers a vernalization requirement in this species. FRI functions via an unknown biochemical mechanism to transcriptionally up-regulate *FLC* (Michaels and Amasino, 1999; Sheldon *et al.,* 1999). The latter is a MADS-box transcription factor that quantitatively represses flowering by repressing the expression of floral-pathway integrators (Michaels and Amasino, 1999). In vernalization-requiring Arabidopsis, the *FLC* transcript accumulates to high levels and this inhibits the photoperiodic-inductive signals. Rapid-cycling accessions of Arabidopsis flower early due to the presence of natural loss-of-function *FRI* or *FLC* alleles. (Johanson *et al.,* 2000). For example, the weak allele of *FLC* in L*er* results from the insertion a transposable element in the first intron (which has a known function in the regulation of *FLC* expression) (Gazzani *et al.,* 2003; Michales *et al.,* 2003).

In Arabidopsis, vernalization promotes flowering through stable epigenetic repression of *FLC.* The establishment and maintenance of silenced chromatin at the *FLC* locus has been associated with a series of covalent modifications introduced at both DNA and histones (Sung and Amasino, 2004; Bastow *et al.,* 2004). Three genes that regulate the *FLC* chromatin state during vernalization, have been found in screens for mutants that unable to flower early after a long-term exposure to cold: *VERNALIZATION 1* (*VRN1*), *VRN2,* and *VERNALIZATION INSENSITIVE 3* (*VIN3*). *VIN3* is specifically expressed upon longer exposure to cold and appears to function in the histone deacetylase complex that modifies *FLC* chromatin. VRN1 and VRN2 function to maintain the VIN3-mediated repressed state of *FLC* chromatin. The repression of *FLC* is stably maintained when plants resume growth in warm conditions, which allows flowering in response to inductive signals in Spring. This

stable repression lasts the mitotic life of the plant, but is relieved during meiosis via unknown mechanisms (Sung and Amasino, 2004; Bastow *et al.,* 2004).

1.2.9. Autonomous pathway

The autonomous pathway has been defined based on the phenotype of mutants that were late flowering under both long- and short-day conditions, compared to the behavior of the wild type (Koornneef *et al.,* 1991). Vernalization or exposure to far-red light accelerates flowering in these mutants (Koornneef *et al.,* 1991). The autonomous pathway constitutes a group of at least seven genes: *FVE, FLOWERING LOCUS D* (*FLD*)*, LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS K* (*FLK*), *FY, FCA,* and *FPA.* The autonomous pathway acts in parallel to *FRI* to negatively regulate *FLC* expression. Thus, similar to *FRI*-carrying plants, mutants in this pathway, are late flowering due to the accumulation of the *FLC* transcript. Though the autonomous mutants have an apparently similar phenotype, genetic studies suggest that they do not function in one simple linear pathway (Henderson and Dean, 2004). Based on double mutants analyses, two epistatic groups were established: *FCA* and *FY* form one group, while the second group consists of *FPA* and *FVE*. *ld* and *fld* mutations were excluded from the epistasis analyses, because they are suppressed by the *FLC* allele in L*er (*Koornneef *et al*., 1998). The *FCA*, *FY* epistatic group has recently been explained on the molecular level, when it became clear that their gene products interact and likely function in one complex. *FCA* encodes a plant-specific protein that in addition to two RNA-recognition motif (RRM) domains, contains C-terminal WW protein interaction domain. This domain interacts with the C-terminal domain of FY, which appears to be an essential component of the RNA 3'-end processing complex (Simpson *et al.,* 2003). Surprisingly, it has recently been demonstrated that FCA is an ABA receptor and that ABA causes dissociation of FCA-FY complex (Razem *et al*., 2006). The relevance of this finding with regard to flowering needs to be further investigated.

Interestingly, two more autonomous genes (*FPA* and *FLK*) also encode proteins that contain RNA-binding domains (Schomburg *et al*., 2001; Lim *et al.,* 2004). An attractive hypothesis is that these proteins regulate *FLC* expression through direct binding and processing of *FLC* mRNA, but so far there is no evidence to

support this. Chromatin modification seems to be another way by which autonomous genes repress *FLC*. FVE and FLD show similarity to components of the mammalian histone deacetylase (HDAC) complex, and in *fld* and *fve* mutants increased levels of histone acetylation of *FLC* chromatin were detected. Other autonomous mutants do not exhibit enhanced histone acetyletion levels. These findings indicate that FLD and FVE likely act together in the HDAC complex that deacetylates histones in the *FLC* chromatin, thus leading to its repression (He *et al.,* 2004; Ausin *et al*., 2004).

LD encodes a homeodomain protein that is targeted to the nucleus. *LD* is strongly expressed in young, rapidly dividing tissues, in particular in the shoot and root apex, which overlaps with the expression pattern described for *FLC* (Lee *et al*., 1994). Though *LD* was the first autonomous gene to be cloned, its biochemical function remains unknown. LD shows no strong similarity to any characterized protein. It has two bipartite nuclear localization sequences, a region of restricted similarity to the homeodomain within the N-terminus, and a glutamine-rich stretch in the C-terminus (Lee *et al*., 1994). Moreover, a comparison of the maize and Arabidopsis LD protein sequences revealed conservation within amino-acid positions, which are responsible for interaction with DNA in the yeast Mata1 homeodomain protein. These features suggest that LD might be a transcription factor, but so far no sequence-specific binding of LD to DNA has been detected (van Nocker *et al.,* 2000). Alternatively, LD may function as a RNA-binding protein to regulate gene activity at the posttranscriptional level, as was demonstrated for the Drosophila homeodomain protein Bicoid (Aukerman *et al.,* 1999).

1.2.10. Activators of *FLC*

Screens for early-flowering mutants under short day photoperiods, and suppressor of the winter-annual habit in *FRI*-containing lines background, led to identification of many genes, encoding proteins that activate *FLC* expression. These genes include *VERNALIZATION INDEPENDENCE3 (VIP3), VIP4, VIP5, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1), FRIGIDA-LIKE1 (FRL1), FRL2, EARLY FLOWERING7 (ELF7), ELF8*, *AERIAL ROSETTE1 (ART1), EARLY FLOWERING IN SHORT DAYS (EFS)* (Zhang *et al.,* 2003; Zhang and van

Nocker, 2002; Noh and Amasino, 2003; Michaels *et al*., 2004). As mentioned above, *FRI* is the key activator of *FLC* expression that determines the winter-annual habit in Arabidopsis. *FRL1, FRL2,* and *ART1* have been shown to be specifically required for the *FRI*-mediated accumulation of *FLC* transcript (Michaels *et al.,* 2004; Poduska *et al.,* 2003). Two *FRI*-like genes have been shown to be required for the *FRI*-mediated upregulation of *FLC* (Michaels *et al.,* 2004).

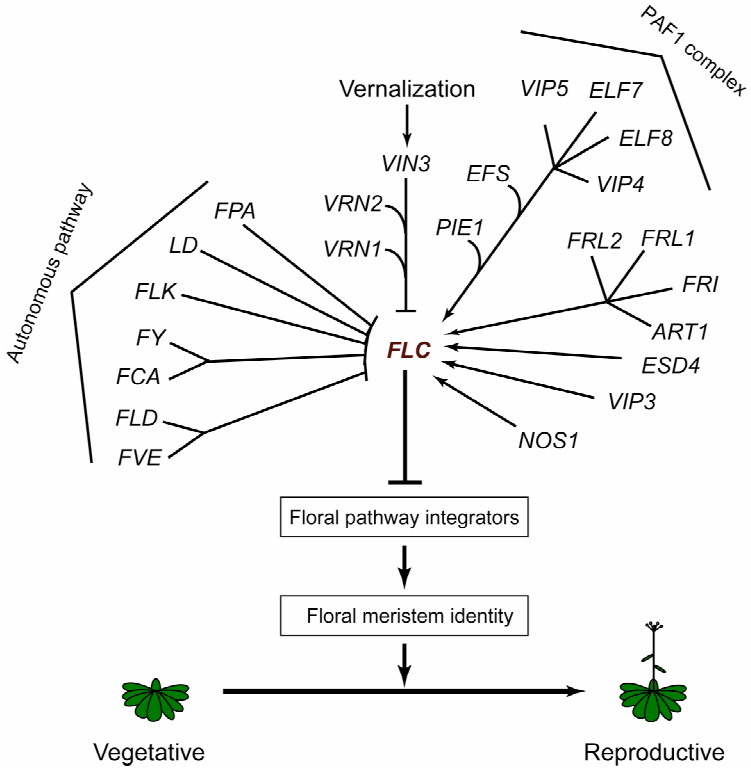

Figure 1.3. Factors that regulate flowering time through the control of *FLC* expression. FLC is a MADS-box transcription factor that quantitatively represses flowering by repressing the expression of floral pathway integrators. The autonomous pathway constitutes a diverse group of at least seven genes: *FVE, FLD, LD*, *FLK*, *FY, FCA,* and *FPA* that act to negatively regulate *FLC* expression. *VIN3*, *VRN1*, and *VRN2* function in the vernalization pathway to downregulate *FLC* expression. The PAF1 complex in turn, possibly associate with RNA polymerase to activate *FLC* transcription. *EFS* and *PIE1* are other components required for active *FLC* chromatin. *FRI* is a major determinant of a winter-annual habit, it upregulates *FLC* expression through an unknown mechanism. *FRL1, FRL2* and *ART2* are specifically required for the *FRI*-mediated activation of *FLC* expression. *EDS4* encodes a SUMO-directed protease, whose one of the functions, perhaps and indirect one, is to activate *FLC*. *VIP3* also promotes *FLC* expression, possibly as a part of un unidentified protein complex. *NOS1* represents a NO-mediated regulation of *FLC* levels. Modified from He and Amasino (2005).

In natural-variation studies, a dominant allele of *ART1* was isolated from the extremely late-flowering accession. *ART1* act synergistically to *FRI* to activate *FLC* expression, which causes the extreme delay in the onset of flowering in Sy-0 (Poduska *et al.,* 2003).

Some of the identified floral activators (*VIP3, ELF7, ELF8, VIP4, PIE1, EFS*) are required for high levels of *FLC* expression, both in *FRI*-containing lines and in autonomous mutants. ELF7, ELF8, VIP4, VIP5 appear to function in the Arabidopsis PAF1-like complex (Zhang et al., 2003; He et al., 2003). In yeast, the PAF1 complex has been shown to activate gene expression by mediating trimethylation of histone H3 at lysine 4 (H3-K4). The complex associates with the RNA polymerase II complex during transcription and recruits the H3-K4 methyltranferase SET1 to target a subset of genes for activation (Ng *et al*., 2003). In Arabidopsis, *elf7* and *elf8* exhibit reduced trimethylation at H3-K4 of *FLC* chromatin and lower levels of *FLC* mRNA, compared to wild-type plants, indicating that the Arabidopsis PAF1 complex regulates expression of *FLC* through an epigenetic mechanism (Zhang et al., 2003; He et al., 2003). Interestingly, *EFS* has been identified as a putative histone H3 methyl transferase that is required for trimethylation at H3-K4 in *FLC* chromatin (Soppe *et al.,* 1999; He and Amasino, 2005). An additional step in the control of H3-K4 trimethylation requires the activity of PIE1, the Arabidopsis relative of ISW1p (a yeast ATP-hydrolyzing, chromatin-remodelling protein). In yeast ISW1p binds di- and trimethylated H3-K4 likely to further modify specific chromatin regions. Exisiting H3-K4 methylation is a prerequisite for ISW1p to bind chromatin *in vivo* (Noh and Amasino, 2003). Taken together, it appears that *VIP5, ELF7, ELF8, VIP4, PIE1, EFS* function in a sequential order to activate the expression of *FLC* through regulation of H3K4 trimethylation.

Interestingly, the PAF1-like complex also seems to be required for the expression of *FLC*-related genes (He et al., 2003). In Arabidopsis, there are five close homologues of *FLC,* called *MADS AFFECTING FLOWERING1* (*MAF1*)/*FLM* to MAF5. *FLM,* and likely *MAF2* are floral repressors acting under non-inductive photoperiods. *MAF3* and *MAF4* might also function to repress flowering, whereas *MAF5* might act as a promoter of flowering, since its expression is induced during vernalization (Scortecci *et al.,* 2001; Ratcliffe *et al*., 2003; Ratcliffe *et al*., 2001).

Another activator of *FLC* expression is *EARLY IN SHORT DAYS 4* (*ESD4*). *ESD4* encodes a nuclear protease that may process a precursor of SMALL UBIQUITIN-RELATED MODIFIER (SUMO) to its mature form. The *esd4* mutant contains lower levels of free SUMO, which results in an early flowering phenotype (Murtas *et al.,* 2003; Reeves *et al.,* 2002). However, it remains to be investigated which of the *FLC* regulators is modified by SUMO and how this affects the activity of the modified protein.

Numerous genes have been found to activate or repress *FLC* through different mechanisms at different stages of plant development (Fig. 1.3). It has been proposed that the activators function early in development to increase levels of *FLC* mRNA, thereby preventing precocious flowering when the plant has not yet accumulated enough resources to successfully complete its reproductive development. Later in development, *FLC* is repressed by the activity of the autonomous or the vernalization pathways and remains low until gametogenesis or early embryogenesis, when the *FLC* repression is reset to allow the start of a new developmental cycle. Thus, it seems that many environmental and endogenous signals interact to precisely control *FLC* expression, ensuring an optimal timing of the transition from the vegetative to the reproductive phase.

1.2.11. Integration of flowering pathways

Distinct genetic pathways regulate the floral transition in response to various environmental and endogenous stimuli. The integration of signals from separate pathways occurs on a small number of genes, termed floral integrators: *FT, SOC1* and *LFY* (Simpson and Dean, 2002). Individual pathways differentially control floral integrators, however, a single integrator may not be regulated by every pathway. For example, *FT* and *SOC1* are immediate targets of CO (*i.e.* the photoperiod pathway) that activates *FT/SOC1* expression (Samach *et al.,* 2000, Hepworth *et al*., 2002) The autonomous and the vernalization pathways are integrated on the floral repressor *FLC* (Michaels and Amasino, 1999, Sheldon *et al.,* 1999). *FLC* delays flowering by repressing the expression of *FT* and *SOC1*, but not *CO*, indicating that the day-length pathway is clearly distinct from the autonomous/vernalization pathways until the

CO/FLC level and converge on the common downstream components (Hepworth *et al*., 2002). Similarly, GA pathway activates expression of *SOC1*, *LFY,* and might activate *FT* (Blazquez *et al.,* 1998; Moon *et al.,* 2003; Gomez-Mena *et al*., 2001; Pineiro *et al.,* 2003). Moreover, both the GA and photoperiod pathways regulate *LFY* expression through separate cis element in the *LFY* promoter (Blazquez and Weigel, 2000), but contrary to *FT* and *SOC1*, *LFY* is not a direct target of CO. *FT*, *SOC1* and *LFY* cannot be the only floral integrators, as the triple *lfy ft soc1* mutant can still flower under long days (Moon *et al.,* 2005). The floral integrators upregulate the expression of the floral meristem identity genes *APETALA1* (*AP1*), *APETALA2* (*AP2*), *FRUITFULL* (*FUL*), *CAULIFLOWER* (*CAL*), and *LFY,* which convert a vegetative to a floral meristem (Boss *et al*., 2004).

The appropriate flowering time requires not only temporal, but also spatial integration of promotive signals. Classical studies have shown that the leaves of a photoperiodically induced plant can evoke flowering when grafted to a non-treated plant. This led to the hypothesis that there is an unknown mobile signal florigen that moves from the leaf to the shoot apex in response to the floral promotive cues (Bernier *et al.,* 1993). For more than 50 years the chemical nature of florigen remained elusive, but very recent studies suggested that *FT*, at least partially, functions as a mobile floral inducing signal (Huang *et al*., 2005; Wigge *et al.,* 2005; Abe *et al.,* 2005). It remains unclear whether *FT* mRNA or FT protein is transported to the shoot apex, and whether *FT* is itself a florigen, or function as a shuttle for the florigen hormone.

1.2.12. *LEAFY*

LFY is an essential regulator in the transition from vegetative to reproductive growth. The *LFY* promoter has been shown to be activated early under long-day conditions, and this correlates with early flowering (Blazquez *et al.,* 1997). Under short days, in contrast, *LFY* promoter activity increases gradually, which is reflected in a delay of the floral transition. Exogenous application of GAs, which accelerate flowering under short days, increases *LFY* promoter activity, as assayed with *LFY::GUS* activity (Blazquez *et al*., 1998). Therefore, it seems that there is a certain

threshold of *LFY* expression sufficient to drive the floral transition. Moreover, a change in the copy number of endogenous *LFY* affects the number of leaves produced before the first flower appears, and hence affects the time of flowering (Blazquez et al., 1997). Expression of *LFY* under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S) leads to early flowering in Arabidopsis and induces flowering under short days in the otherwise non-flowering *ga1-3* mutant. These findings further support that *LFY*, at least partially, functions as a flowering-time gene. The expression of *LFY* precedes the expression of other meristem-identity genes, which are specifically expressed in flowers. *LFY* is necessary for secondary flower-meristem formation from the primary inflorescence meristem, and later in development it plays a role in flower patterning (Weigel *et al.,* 1992; Blazquez *et al.,* 1997). Thus, *LFY* posseses the properties of both a flower meristem-identity gene and a flowering-time gene.

1.3. Brassinosteroids

The first identified brassinosteroid (BR), brassinolide (BL), was isolated from *Brassica napus* pollen in 1979 and was demonstrated to promote cell elongation in various bioassays in a range of plant species (Grove *et al.,* 1979). Brassinosteroids are polyhydroxylated steroids, structurally similar to animal steroid hormones such as androgens, estrogens, and corticosteroids (Fig. 1.4). BRs can be detected widely in the plant kingdom including algae, ferns, gymnosperms, and angiosperms. At present, more than 50 naturally occurring BRs were identified in plants (Clouse and Sasse, 1998; Clouse, 2002, Vert *et al.,* 2005). Brassinolide (BL) is believed to be the most bioactive BR in Arabidopsis, but in some plants, castasterone, an immediate precursor of BL, functions as a main BR. BRs are ubiquitously synthesized and the highest levels are detected in pollen and mature seeds, but young growing tissues contain significant amounts of these phytohormones (Adam et al., 1996). BRs are plant-growth promoters that regulate stem elongation, pollen tube growth, photo- and skotomorphogenesis, xylogenesis, cell elongation and differentiation, and influence responses to biotic and abiotic stress. BR-deficient mutants have been identified as

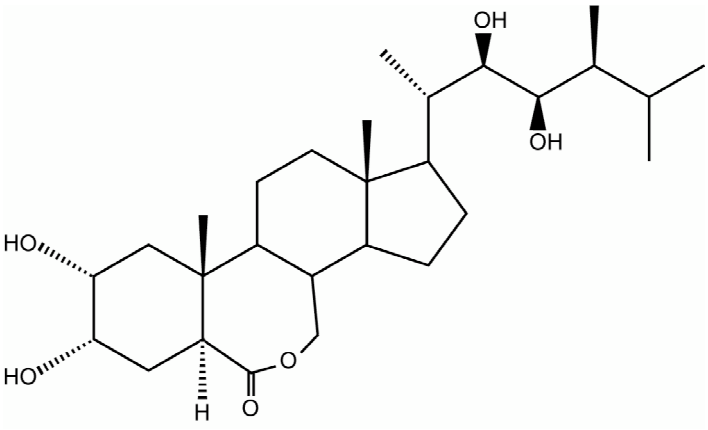

Figure 1.4. The structure of brassinolide (BL), which is the most biologically active brassinosteroid in Arabidopsis. BL is polyhydroxylated plant steroid hormone that is structurally similar to cholesterol-derived animal steroid hormones.

dwarf plants in many plant species, such as Arabidopsis, rice, japanese morning glory, pea, and tomato (Bishop and Koncz, 2002). Other specific features of BR-deficient mutants are dark green leaves, reduced fertility, altered vascular development and prolonged life-span. They also exhibit light-grown phenotype, both on physiological and molecular levels, when grown in darkness, indicating the role of BRs in light signalling (Schumacher and Chory, 2000; Bishop and Koncz, 2002; Haubrick and Assmann, 2006).

The BL-biosynthethic pathway was initially established using cells of *Catharanthus roseus* (Fujioka and Sakurai, 1999; Sakurai, 1999). The identification of Arabidopsis various BR-deficient mutants allowed confirmation and further characterization of the BL-biosynthetic pathway. All plants sterols seems to be synthesized from cycloartenol, which is derived from squalene. BL appears to be synthesized from campasterol via teasterone, typhosterol and castasterone (with several intermediate steps) by two parallel branched pathways: the early or the late C-6 oxidation pathway, depending when C-6 oxidation happens early (before) or late (after) hydroxylation of the side chain. The final step is oxidation of castasterone to brassinolide (Yokota, 1991, 1997)(Fig. 1.5).

Two enzymes, DWF4 and CPD seem to be candidates for rate-limiting steps in BL biosynthesis, because their transcription is under the control of a negative BR-feedback loop (Vert *et al.,* 2005). Both proteins are cytochrome P450 steroid

hydroxylases that catalyse the conversion of 6-oxocampestanol/campestanol to cathasterone/6-deoxocathasterone and cathasterone/6-deoxocathasterone to teasterone/6-deoxoteasterone, respectively (Szekeres *et al*., 1996; Choe *et al*., 2001). Another way of maintaining BR-homeostasis is conversion of BRs into their inactive forms by epimerisation, followed by glucosylation, esterification, and hydroxylation (Clouse, 2002, Bishop and Koncz, 2002; Haubrick and Assmann, 2006).

1.4. BRI1-BR receptor

Brassinosteroids are perceived by the plasma-membrane localized receptor BRI1. BRI1 is a leucine-rich repeats receptor-like kinase (LRR-RLK) that consists of 24 LRR repeats, interrupted by the 70 amino-acid island, followed by a transmembrane domain and a cytoplasmic serine/threonine kinase domain (Li and Chory, 1997) (Fig. 1.6).

Several experiments demonstrated that BRI1 is a critical component for BR binding and perception. These include: i) *BRI1* overexpression leads to increase in the number of BL binding sites and the BL-binding activity can be immuno-precipitated with antibodies against a tagged BRI1, ii) in competition experiments, binding affinities of BR-binding sites correlate with the bioactivity of the respective brassinosteroid, iii) a chimeric protein consisting of the extracellular domain of BRI1 fused to the kinase domain of a rice LRR-RLK, XA21 involved in pathogen defense, induced defense responses after BL-application, iiii) direct binding of BL to BRI1 was demonstrated with native and recombinant proteins. Experiments with the recombinant protein containing only the island domain and the neighbouring C-terminal LRR repeat revealed that that part of BRI1 is sufficient to bind radioactive BL with an affinity similar to the affinity observed with the full-length receptor, indicating that this region of BRI1 is responsible for binding of BL (Wang *et al.,* 2001; He *et al.,* 2000; Kinoshita *et al.,* 2005)

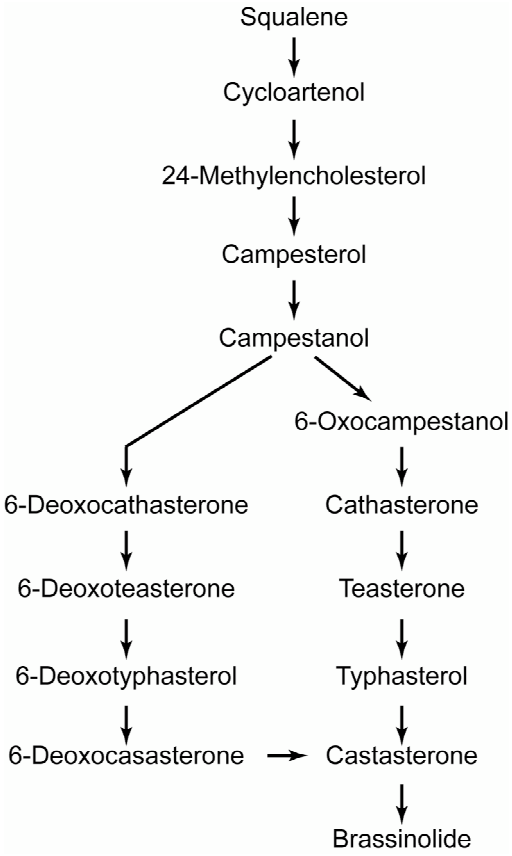

Figure 1.5. Major steps in sterol biosynthesis in plants. Campesterol is the starting point for brassinolide-specific biosynthetic pathway. Campesterol is reduced to campestanol, which is further converted to castasterone, in two parallel branched pathways: the early or the late C-6 oxidation pathway. Castasterone is an immediate precursor of brassinolide. Adapted from "Plant Growth and Development. Hormones and Environent", Ed. LM Srivastava (2001).

Binding of BL initiates a signal-transduction cascade that transduces the signal through the membrane into the cell and evokes an array of BL-induced responses. *BRI1* was found in many independent genetic screens for BR-insensitive mutants suggesting that it is the major BR-receptor in Arabidopsis (Clouse *et al*., 1996; Li and Chory, 1997). Recently, two BRI1-like proteins, BRL1 and BRL3 were identified to likely function as BRs co-receptors. However, their expression is restricted to vascular cells, while BRI1 is ubiquitously expressed in all growing cells (Cano-Delgado *et al.,* 2004; Zhou *et al.,* 2004). Thus, *BRI1* encodes a main receptor for BRs in Arabidopsis. This notion is further supported by a phenotype of *bri1* that resembles the strongest BR-biosynthetic mutant Clouse *et al*., 1996; Li and Chory, 1997). These

phenotypes include light-grown features in the light and in the darkness, extremely dwarf morphology, reduced apical dominance, reduced fertility, impaired vascular development, and delayed senescence (Li and Chory, 1997).

Interestingly, the BRI1 ortholog in tomato was shown to bind both BRs and systemin. Systemin is a small peptide involved in plant defence, present only in the Solaneae subtribe of the Solanaceae family, including tomato and potato (Sheer and Ryan, 1999, 2002; Montoya *et al.,* 2002). These findings raise possibilities that the Arabidopsis BRI1 could also function as a receptor for ligands other than BL, *eg.* small peptides. However, to date there is no evidence to support broader ligand-binding specificity of the Arabidopsis BRI1. Future work should clarify whether AtBRI1 has a pleiotropic receptor role.

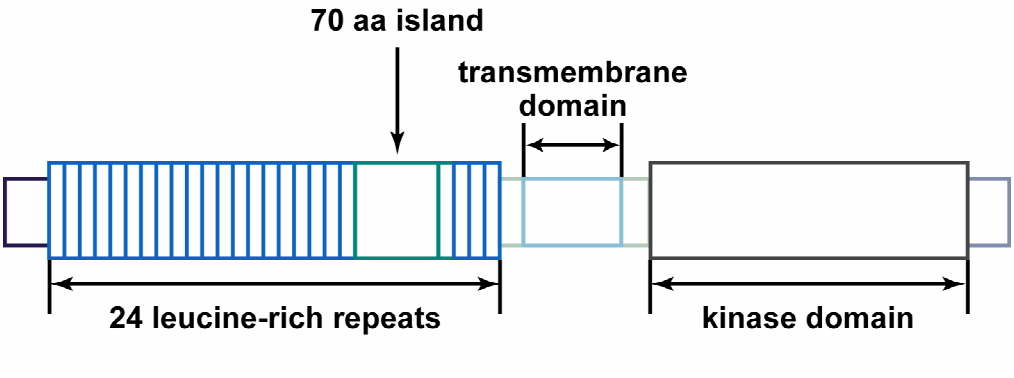

Figure 1.6. A domain structure of the BR-receptor, BRI1. BRI1 is a transmembrane leucine-rich-repeats receptor serine/threonine kinase (LRR-RLK). BRI1 consists of the extracellular 24 LRR rpeats interrupted by a stretch of 70 amino-acid termed the island domain, followed by a transmembrane region and an intracellular kinase domain. BL binds to the "island domain" and the neighbouring C-terminal LRR repeat. Adapted from Vert *et al*. (2005).

1.5. Current model for BR signalling

The BR-signaling cascade is initiated at the plasma membrane by activating BRI1. In the absence of BRs, BRI1 is found as inactive homo-oligomers, in a basal activity state with the kinase domain being hypophosporylated and autoinhibited by the carboxyterminal (CT) region through cis- or trans- repression (Wang *et al.,* 2005

a, b). After BL binding to the extracellular domain of BRI1, conformational changes are induced which results in trans-phosphorylation of the CT region within the dimer. This phosphorylation might enhance activity of BRI1 kinase and promote further phosphorylation creating a fully activated receptor (Wang *et al.,* 2005 a, b). Subsequently, BRI1 forms a multimeric complex with another LRR RLK, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) through their intracellular domains (Li *et al.,* 2002; Nam and Li, 2002, Kinoshita *et al.,* 2005, Wang *et al.,* 2005a). This is thought to transduce BR signal. In support of this, BAK1 is not required for BR binding to BRI1, but ligand binding to BRI1 induces hetero-oligomerization of BRI1 and BAK1 and phosphorylation of both proteins (Li *et al.,* 2002; Nam and Li, 2002, Kinoshita *et al.,* 2005, Wang *et al.,* 2005a). The next known downstream component in the BR-signaling is the BRASSINOSTEROID-INSENSITIVE-2 (BIN2), a glycogen synthase kinase-3 (GSK3)-like kinase (Choe *et al.,* 2002; Li and Nam, 2002, Perez-Perez *et al.,* 2002). BIN2 functions as a negative regulator of BR-signaling by phosphorylating two closely related transcription factors BES1 and BZR1 to "tag" them for proteasome-mediated degradation (He *et al*., 2002; Zhao *et al.,* 2002). In addition, a phosphatase, the *bri1* SUPPRESSOR 1 (BSU1) acts on BES1, and likely BZR1 to dephosphorylated them (Mora-Garcia *et al.,* 2004). Under normal growth condition, BES1 and BZR1, are found in both phosphorylated and dephosphorylated forms. Upon BR application, BIN2 is inhibited and/or BSU1 is activated by an unknown mechanism, leading to the accumulation of BES1/BZR1 in their stable hypophosporylated forms in the nucleus (Wang *et al*., 2002; Yin *et al.,* 2002). BES1 forms a dimer with the transcription factor BIM1, and together they bind to the E-box motifs (CANNTG) in the promoters of a subset of BR-responsive genes to activate their transcription. BZR1 in turn, functions as a repressor of the BR-feedback regulated genes (BR- biosynthetic gene *CPD*) by directly binding to the CGTG(T/C)G elements (He *et al.,* 2005; Yin *et al*., 2005). It seems plausible that

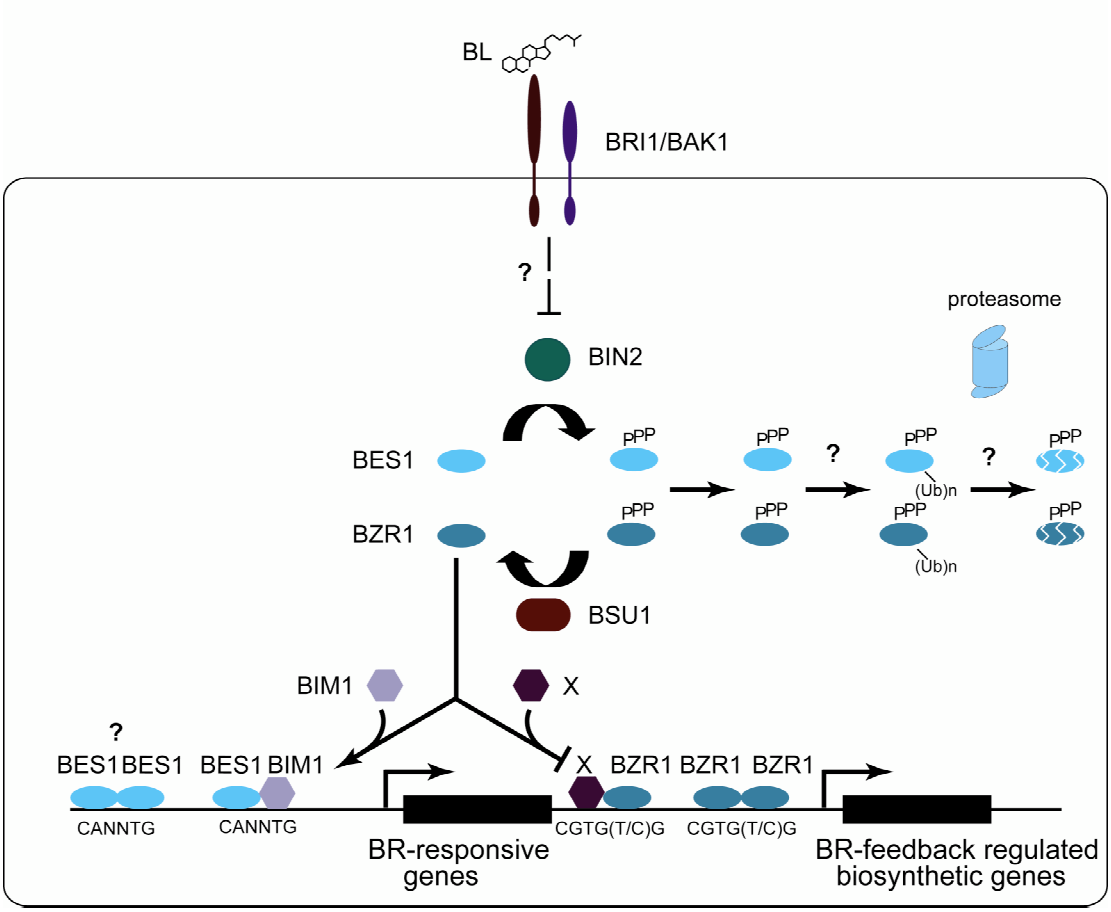

Figure 1.7. A current model for BR signaling. In non-stimulated cells, the BIN2 GSK3 kinase phosphorylates the transcription factors BES1 and BZR1, which likely targets them for ubiquitination, followed by proteasome-dependent degradation. Upon BL binding to the island domain of the plasma membrane-localized receptor, BRI1, BRI1/BAK1 *trans*-phosphorylate initiating signal transduction across the membrane. This leads to inhibition of the BIN2 and/or activation BSU1 by an undetermined mechanism. As a result, BES1 forms a dimer with another transcription factor, BIM1, and they bind to E-box motifs, CANNTG in the promoters of a subset of BR-regulated genes to activate transcription of target genes. BZR1 acts as a repressor of the BR-feedback regulated genes (eg. *CPD*). BZR1 binds directly to CGTG(T/C)G elements, possibly in association with an uncharacterized transcription factor (X). The downregulation of BR-biosynthetic genes upon BL-perception ensures maintaining the BR homeostasis.

BZR1, similarly to BES1, would associate with another protein to bind the specific DNA sequence. Since BRs are believed to act in tissues were they are synthesized, repression of BR-biosynthetic genes is an important part of mechanisms that maintain the BR homeostasis (He *et al.,* 2005; Yin *et al*., 2005, Vert *et al*., 2005). (Fig. 1.7).

1.6. *BRI1* might regulate flowering time

A genetic screen has been preformed in the Davis group to identify novel components modulating the time of floral transition in Arabidopsis. In this screen, the autonomous pathway mutant, *luminidependens* (*ld*) (Lee *et al.,* 1994) was EMS-mutagenized and enhancers of its late-flowering phenotype were identified.

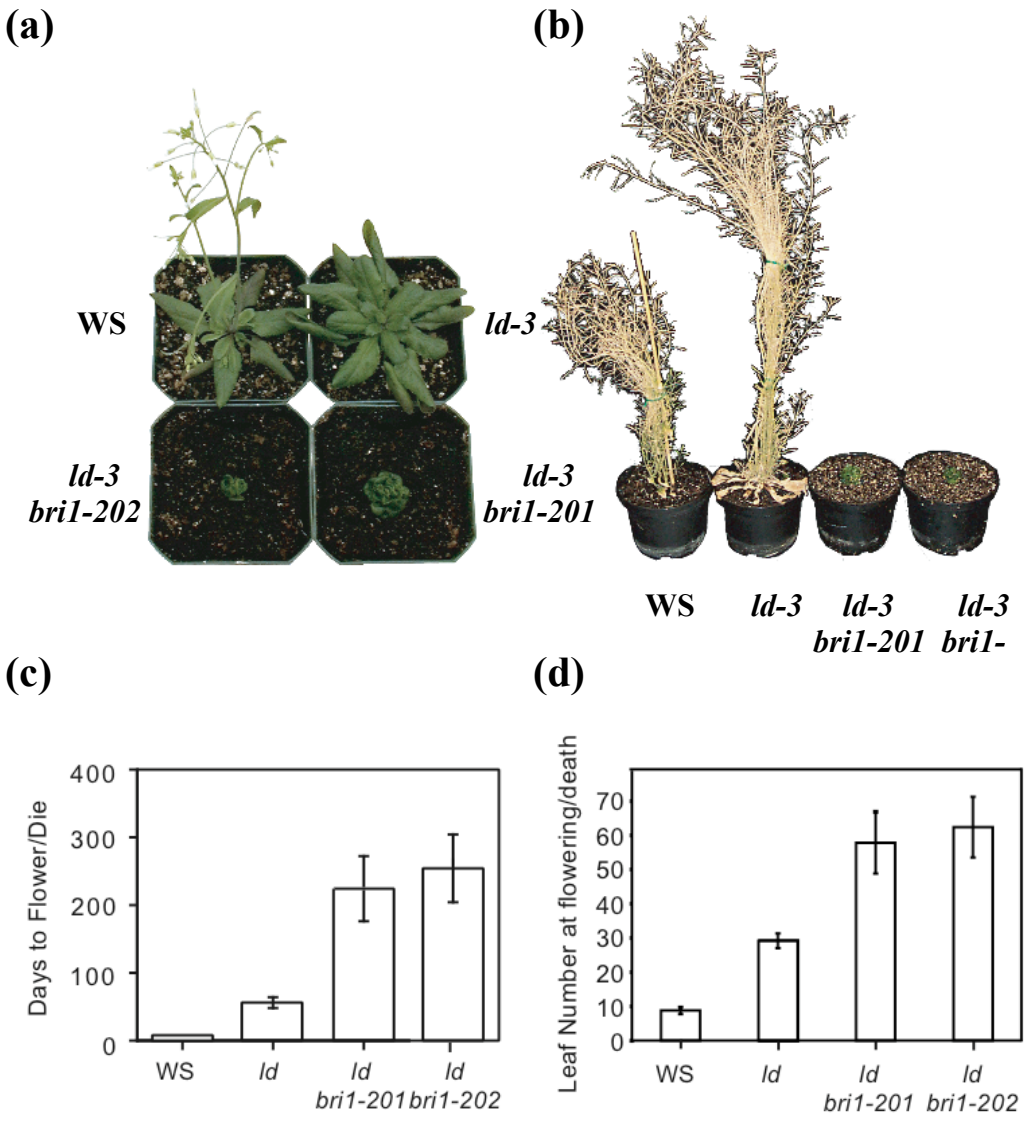

Figure 1.8. Identification of two *bri1* alleles as enhancers of the late-flowering phenotype of the autonomous mutant *ld*. An enhancer screen was performed to isolate the modifiers of the late-flowering phenotype of *ld*. Two independent alleles mutated in the gene encoding the BR-receptor *BRI1* were found to cause extremely late flowering in the *ld* mutant background. Phenotypes of the wild-type WS plants, the single *ld-3* mutants used in the screen and two isolated *bri1-201 ld-3*, *bri1-201 ld-3* double mutants at the age of 2 months (a) and 6 months (b). Flowering time of the described lines as measured by days to flowering (c) and leaf numbers at flowering or death (d).

Two independent alleles of *bri1* were found to lead to the extremely late flowering in the *ld* background. In the presence of functional *LD,* however, isolated mutants in both *bri1* alleles have only weak flowering phenotypes (Fig. 1.8, 1.9). As described above, *BRI1* encodes a LRR-RLK kinase that functions as the BR-receptor (Wang *et al.,* 2001; He *et al.,* 2000; Kinoshita *et al.,* 2005), thus the result of the screen indicated that *BRI1* or BRs play a role in regulating flowering time in Arabidopsis.

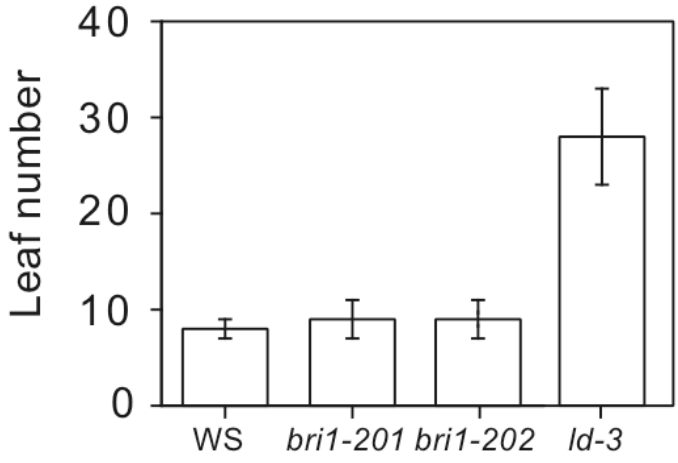

Figure 1.9. Flowering time of the wild-type WS plants, the single *bri1-201*, *bri1-202* and *ld-3* mutants under long days. Flowering time was scored as the total leaf number at flowering. The single *bri1* mutants have only mild late-flowering phenotype compared to WS and to the much later flowering *ld* mutant.

1.7. The goals of this study

The aim of this thesis was to define and compare the roles of BR-receptor BRI1 and BRs in floral transition. The rationale underlying this goal was a previously performed genetic screen, in which two alleles of *bri1* were found to enhance strongly the late-flowering phenotype of the autonomous mutants *lumindependens* (*ld*) (Davis, unpublished). Genetic and molecular-genetic approaches were used, to place *BRI1*and BRs in the flowering-genetic network. Various double mutant combinations between *bri1* and known flowering-time mutants were constructed and their flowering time

was analyzed. Additionally, expression of key flowering-time genes was examined in these plants. Furthermore, the role of BRs and *BRI1* in the control of flowering time was evaluated by comparable analyses of flowering behaviors of BR-deficient mutant *cpd* and *bri1*. The role of BRs in floral transition was further investigated in the context of their possible interactions with the GA- and ABA-regulated pathways. Double-mutant combination deficient in BR, ABA, and GA were constructed and their flowering time, as well as, expression of key flowering-time genes were examined. Furthermore, transgenic plants that overexpress *DWF4, NCED3,* and *GA5,* leading to BR-, ABA- and GA- overproducing phenotypes, respectively, were included in these studies. Finally, attempts were made to test whether the BR-pathway converge on the *LFY* promoter, which is one of the described integration points in the flowering-regulating network (Blazquez *et al.,* 2000). For this purpose, the *LFY::LUC+* reporter system was applied.

2. Materials and Methods

2.1. Materials

2.1.1.Chemicals

Laboratory grade chemicals were purchased from Amerscham Biosciences (Freiburg), Merck (Darmstadt), Fluka (Neu-Ulm), Sigma-Aldrich (Taufkirchen), Serva (Heidelberg), Duchefa (Haarlem, Netherlands), and Roth (Karlsruhe).

2.1.2. Enzymes

Restriction enzymes were purchased from New England Biolabs (Frankfurt a.M.) and Fermentas (St. Leon-Rot).

Other enzymes:

Taq DNA polymerase	Peqlab (Erlangen)
Pfu Turbo HotStart DNA polymerase	Stratagene (Heidelberg)
T4 DNA ligase	Fermentas (St. Leon-Rot)
Klenow enzyme	Roche (Mannheim)
DNaseI	Roche (Mannheim)
RNaseH	Fermentas (St. Leon-Rot)
Superscript II RT	Invitrogen (Karlsruhe)
BP-clonase (GATEWAY®-Technology)	Invitrogen (Karlsruhe)
LR-clonase (GATEWAY®-Technology)	Invitrogen (Karlsruhe)

2.1.3. Bacterial strains

2.1.3.1. *E. coli*

DH5 (Invitrogen, Karslruhe):

DB3.1(Invitrogen, Karslruhe):

XL10-Gold (Stratagene, Heidelberg)

2.1.3.2. Agrobacterium tumefaciens

ABI: C58 strain with pMP90RK (Koncz and Schell, 1986)

GV3101 with pMP90RK (Koncz and Schell, 1986)

2.1.4. Plants

All *Arabidopsis thaliana* (Arabidopsis) lines were are in the Ws-2 (WS) background, except for *ga1-3*, which is in the L*er* background.

Listed below are Arabidopsis mutants used in this studies:

Genotype	reference
ld-2	Lee *et al.,* 1994
ld-3	Lee *et al.,* 1994
bri1-4	Noguchi *et al.,* 1999
gi-11	Fowler *et al.,* 1999
cpd-10	Obtained from F. Tax, University of Arizona, Tucson (unpublished, nr 393a from F. Tax collection, for purposes of this thesis named *cpd-10*)
fca-11	Obtained from R. Amasino, University Wisconsin, Madison
FRI^{SF2},	Obtained from R. Amasino, University Wisconsin, Madison, the WS line containing the *FRI* gene introgressed from the ecotype San Felieu
ga1-3	Sun *et al.,* 1992
aba2-2	Cheng *et al.,* 2002

2.1.5. Vectors

pDONR207	Cloning vector for the GATEWAY® system (Invitrogen, Karslruhe)
pLeela	GATEWAY®-compatible plant expression to express a DNA fragment under the control of CaMV35S promoter (Marc Jacoby, MPIZ)

pJawohl8-RNAi	GATEWAY®-compatible plant expression vector for RNAi (Bekir Ülker, MPIZ)
pPZP221-*Luc*+NosT	Plant expression vector containing *LUCIFERASE+* as a reporter gene, suitable for promoter activity studies (Millar laboratory, Warwick University, UK)

2.1.6. Oligonucleotides

Oligonucleotides were synthesized by Invitrogen (Karlsruhe) and Sigma (Steinheim). Listed below are oligonucleotides used for plant genotyping:

Primer name	Sequence (5' → 3')
FRIforw	CAGATTTGCTGGATTTGATAAGG
FRIrev	GAAATTCACCGAGTGAGAACAGA
FLCLerfor	AAACAATCTGGACAGTAGAGGCTTAT
FLCLerrev	CAGGCTGGAGAGATGACAAAA
gin11RT	AGTGGCATTGATCACTGGAG
gin11revRT	GTGAATCCTCCATCAATCATC
aba23rev	TCTTCTCCGGTATCATTACACG

Listed below are oligonucleotides used for cloning:

Gene	Primer	Sequence (5' → 3')
DWF4	dwf4oxl	(GWF)CCATGTTCGAAACAGAGCATCA
	dwf4oxr	(GWR)TTACAGAATACGAGAAACCCTAATA
NCED3	nced3oxl	(GWF)CCATGGCTTCTTTCACGGCAACG
	nced3oxr	(GWR)TCACACGACCTGCTTCGCCA
GA5	ga5oxl	(GWF)CCATGGCCGTAAGTTTCGTAACAA
	ga5oxr	(GWR)TTAGATGGGTTTGGTGAGCCAA
FLC 5'UTR	FLC5UTRfor	(GWF)CCCGAGAAAAGGAAAAAAAAAAATA
	FLC5UTRrev	(GWR)CGGCTTCTCTCCGAGAGGG
LFY promoter	5LFYAPROM	CACTACCTGTCGACCAGAGAAGAAAAAAACAG
	3LFYAPROM	CTTCAGGCCCGGGAATCTATTTTTCTCTCTC

GWF denotes GGGG*att*B1 site, GWR denotes GGGG*att*B2 site, (GATEWAY®)

Listed below are oligonucleotides used for RT-PCR :

Gene name	Primer name	Sequence (5' → 3')	T [°C]	n
EF1α	EF1alfapl	GTTTCACATCAACATTGTGGTCATTGG	60	17-22
	EF1alfapr	GAGTACTTGGGGGTAGTGGCATCC		
FT	FTRTPCRfor	ACTATATAGGCATCATCACCGTTCGTTACTCG	58	23-28
	FTRTPCRrev	ACAACTGGAACAACCTTTGGCAATG		
SOC1	SOC1RTfor	GAACAAATTGAGCAGCTCAAG	58	23-28
	SOC1RTrev	GCAGCTTTAGAGTTTTGTTAC		
FLC	FLC4ex	GCTTGTGGGATCAAATGTCA	56	20-28
	FLC5ex	AACAAGCTTCAACATGAGTTCG		
LFY	LFY1ex	CTAGACGCCGTCATTTGCTA	56	35
	LFY2ex	CGCATCAGTCTGGTCTTGTT		
DWF4	DWF41750	TCCCTAGTGGGTGGAAAGTG	56	25
	dwf4end	TTACAGAATACGAGAAACCCT		
NCED3	nced31150	CAAGATTCGGGATTTTAGACA	56	25
	nced3oxr	(GWR)TCACACGACCTGCTTCGCCA		
GA5	GA5pl	AAGGCCTTTGTGGTCAATATCGGC	56	25
	ga5end	TTAGATGGGTTTGGTGAGCCAA		
MAF1	MAF1FLMRTfor	ATGGTCTCATCGACAAAGCTCGAC	56	25
	MAF1FLMRTrev	CTCTTAATTATGAATCAGGCTTTGAG		
MAF5	MAF5-RT-for	GGGGATTAGATGTGTCGGAAGAGTGAAG	60	28
	MAF5-RT-rev	GATCCTGTCTTCCAAGGTAACACAAAGG		
CO	COpfor	AAACTCTTTCAGCTCCATGACCACTACT	60	30
	COprev	CCATGGATGAAATGTATGCGTTATGGTTA		

n denotes number of cycles used in PCR

2.1.7. Media

Media for bacteria:

Media for bacteria were prepared as described by Sambrook and Russell (Sambrook and Russell, 2001).

When required, antibiotics were supplemented to the following final concentrations:
Ampicillin 100 mg/l
Carbenicillin 100 mg/l
Gentamicin 25 mg/l
Rifampicin 100 mg/l
Kanamycin 50 mg/l
Spectinomycin + Streptomycin 50 mg/l each (100 mg/l in total)

Media for plants:
MS-medium:
2.2 g MS salt, 0.5g MES, 12 g agar, H_2O to 1 l
pH adjusted with KOH to 5.7

For transgenic plants selection, MS-medium was supplemented with antibiotics to the following final concentrations:
Gentamicin 50 mg/l
Phosphinothricin (PPT) 12 mg/l

2.1.8. Solutions

Standard molecular solution and buffers were prepared as described by Sambrook and Russel (2001).
Luciferin stock solution: 50mM beetle D-luciferin in 0.1 M sodium phosphate buffer, pH 8
Luciferin working solution: 10 x diluted stock solution in 0.01 % Triton X-100

2.2. Methods

Standard molecular biological techniques such as DNA, and RNA manipulation were carried out as described by Sambrook and Russell (Sambrook and Russell, 2001)

2.2.1. DNA manipulation

All kits used for nucleic acid manipulation were purchased from Qiagen (Hilden).

2.2.1.1. Preparation of plasmid DNA from E.coli

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit according to manufacturer's instruction.

2.2.1.2. Isolation of plant genomic DNA

Genomic plant DNA was isolated from Arabidopsis flowers or siliques according to Michaels and Amasino (2001). High quality DNA for cloning was extracted by means of DNeasy Plant Mini Kit.

2.2.1.3. Purification of PCR-amplified fragments

PCR products used for cloning or as probes in gene-expression analyses were purified directly from PCR with Qiaquick PCR Purification kit or were first separated on low-percent agarose gel and subsequently extracted using QIAquick Gel Extraction kit according to manufacturer's instructions.

2.2.1.4. DNA sequencing

Sequencing was performed by the MPIZ DNA core facility (ADIS) on Applied Biosystems (Weiterstadt, Germany) ABI Prism 377, 3100 and 3730 sequencers using BigDye-terminator chemistry.

2.2.1.5. PCR

Basic protocol for PCR performed with regular Taq polymerase (PeqLab, Erlangen)
Reaction mix (total volume of 10 µl):

Template DNA	1 µl
Blue Taq polymerase PCR buffer (PeqLab)	1 µl
Enhancer solution (PeqLab)	2 µl
2.5 mM dNTP mix	1 µl
$MgCl_2$ 25 mM	0.8 µl
10µM forward primer	0.25 µl
10µM reverse primer	0.25 µl
dH_2O	3.65 µl
Taq DNA polymerase (5U/µl)	0.05 µl

Thermal profile:

	step	Temperature [°C]	Length of the step
1	Initial denaturation	94	2 min
2	Denaturation	94	15 sec
3	Annealing	55-60	30 sec
4	Extension	72	30 sec-2 min
5	Final extension	72	5-10 min

Steps 2 to 4 were repeated 34-39 times.

Basic protocol for PCR performed with regular *PfuTurbo* HotStart DNA polymerase (Stratagene, Heidelberg)
Reaction mix (total volume of 20 µl):

Template DNA	2 µl
10 x cloned *Pfu* reaction buffer	2 µl
2.5 mM dNTP mix	2 µl
10µM forward primer	0.5 µl
10µM reverse primer	0.5 µl
dH_2O	12.6 µl
PfuTurbo HotStart DNA polymerase (2.5 U/µl)	0.4 µl

Thermal profile:

	step	Temperature [°C]	Length of the step
1	Initial denaturation	95	2 min
2	Denaturation	95	30 sec
3	Annealing	50-60	30 sec
4	Extension	72	30 sec-7 min
5	Final extension	72	10 min

Steps 2 to 4 were repeated 34 times.

2.2.1.6. Cloning

Using the GATEWAY® system.

BP reaction:

attB-PCR product	25 fmol
GATEWAY® BP clonase	1µl
BP reaction buffer (5x)	1µl
Destination vector (150 ng/µl)	0.5 µl
dH_2O	to 5 µl

LR reaction:

Entry clone (50 ng/µl)	1.25 µl
GATEWAY® LR clonase	0.5 µl
LR reaction buffer (5x)	0.5 µl
Destination vector (50 ng/µl)	0.25 µl

Reactions were carried out in room temperature between 1 hour to the overnight incubation.

Ligations were done according to Sambrook and Russell (2001).

2.2.2. RNA manipulation

2.2.2.1. Isolation of total RNA

RNA was extracted using TRIzol Reagent (Invitrogen, Karlsruhe) according to the manufacturer´s protocol. RNA used in RT-PCR was isolated with the RNeasy Plant Mini Kit (Qiagen, Hilden).

2.2.2.2. RNA gel-blot analysis

5-10 µg (as calculated by measuring absorbance at 260 nm) of total RNA was analyzed in one experiment. Prior to loading, RNA was precipitated over night in the presence of 1/10 volume 3M NaOAc and 2.5 volumes absolute EtOH at -20°C. On the following day, RNA was pelleted by 15 min centrifugation at 10.000 rpm at 4°C.

The RNA pellet was washed with 70% EtOH, centrifuged for 5 min 10.000 rpm at 4°C, and dried. The pellet was re-suspended in 24 µl RNA loading mix. After 10 min incubation on ice, the samples were denatured by heating at 95 °C for 5 min, spinned down briefly at 4°C and loaded onto the gel. The RNA ladder (Invitrogen, Karslruhe) was processed in the same way, except for additionally including 0.5 EtBr µl (7mg/ml) into the RNA loading mix. RNA was separated on a 1.5% denaturing agarose gel. The gel was prepared by dissolving 3.45 g of agarose in 200 ml of DEPC-treated dH_2O. The solution was cooled down to 55°C and 22.3 ml of 10 x MOPS and 7 ml of formaldehyde were added. The electrophoresis was performed in 1 x MOPS at 60-100 V. After the run, the gel was washed in dH_2O for 15 min, and twice 10 x SSC for 15 min. The gel was blotted to the Hybond-NX membrane by means of the capillary transfer according to Southern (1975), with 10 x SSC used as the transfer buffer. The transfer was carried out over night at room temperature and the RNA was immobilized to the membrane by crosslinking with UV Stratalinker (set at AUTO, 1200V) and baking for 2 hrs at 80°C. The membrane was prehybridized in Church and Gilbert buffer at 65°C for 1-2 hrs before radioactively labeled probe was added. The probe was labeled as follows:

60-90 ng DNA
dH_2O to 19 µl
denaturation at 95°C for 5 min
+ 3 µl of Hex-mix (10x, Roche)
+ 3 µl of 5 mM dNTPs (without dCTP)
+ 4 µl of α^{32}PdCTP
+ 1µl of Klenow enzyme (2U/µl)
incubation at 37°C, 30 min-6 hrs

After the labeling, the probe was purified to remove unincorporated nucleotides with QIAquick Nucleotide Removal kit (Qiagen, Hilden) according to the manufacturer's instructions. Subsequently, the probe was denatured at 95°C for 5 min, cooled on ice, centrifuged briefly, and was added to freshly changed hybridization buffer. Hybridization was carried out at 65°C, overnight.

After the hybridization, the blot was washed as follows:
First rinse with 2 x SSC 0.1 % (w/v) SDS
incubation with 2 x SSC 0.1 % (w/v) SDS, 40 min
incubation with 1 x SSC 0.1 % (w/v) SDS, 25 min
incubation with 0.5 x SSC 0.1 % (w/v) SDS, 15 min
incubation with 0.1 x SSC 0.1 % (w/v) SDS, 3-5 min

The blot was sealed in foil and exposed to storage phosphor screen. The signal was visualized using a Phosphorimager (Molecular Dynamics) and the signal strengths were quantified using ImageQuant software (Molecular Dynamics).

2.2.2.3. RT-PCR

1 -5 µg of total RNA was used for cDNA synthesis. Prior to the synthesis, RNA was treated with DNaseI to remove any residual genomic DNA. The following procedure was applied :

Total RNA	7.2 µl
5 x RT buffer (Invitrogen)	1 µl
25 mM $MgCl_2$	0.8 µl
DnaseI (10U/µl) (Roche)	1 µl

Incubation at room temperature, 15 min
+ 1 µl of 25 mM EDTA
Incubation at 65°C, 15 min
+ 1 µl of oligo$(dT)_{12-18}$
Incubation at 70°C, 10 min
Incubation on ice, at least 1 min
+ 4 µl of 5 x RT buffer (Invitrogen)
+ 1 µl of 10 mM dNTP mix
+ 2 µl of 0.1 M DTT
+ 0.5 µl of RiboLock Ribonuclease Inhibitor (Fermentas)
Incubation at 45°C, 5 min
+ 1 µl of Superscript II RT (200U/µl)

Incubation at 45°C, 50 – 75 min
Incubation at 70°C, 15 min
Chilling on ice
+ 1 µl of RNaseH (5U/µl)
Incubation at 37°C, 20 min

cDNA was diluted with H_2O to the final volume of 80-100 µl prior to PCR. PCR was carried out in a total volume of 10-20 µl, 1 µl of cDNA was used per 10 µl of reaction volume. Different numbers of PCR cycles depending on the analysed transcript were applied.
PCR products were separated on 2-3 % agarose TBE-gels. The DNA was stained with EtBr and visualized using a Phosphorimager (Molecular Dynamics).

2.2.3. Transformation of *E.coli*

Competent *E.coli* cells were prepared according to Inoue method (Inoue *et al.,* 1990). Cells were transformed by 30 seconds heat shock treatment at 42 °C. 1 µl of plasmid DNA was used to transform 50-100 µl of cells. Transformed bacteria were selected on LB-agar plates containing appropriate antibiotics. Plates were incubated at 37 °C for 24 hours.

2.2.4. Transformation of *Agrobacterium tumefaciens*

Transformation of *Agrobacterium* was done by electroporation according to Wen-Jun and Forde (1989). 1 µl of plasmid DNA was used to transform 50 µl of cells. Transformation was performed at field strength of 12.5 kV/cm, a capacitance of 25 µF and resistance of 400 to 600 ohms. Transformed bacteria were selected on YEBS-agar plates containing appropriate antibiotics. Plates were incubated at 28 °C for 24 to 72 hours.

2.2.5. *Agrobacterium*-mediated plant transformation

Plants were transformed using the floral-dip method (Clough and Bent, 1998). Flowers were dipped in *Agrobacterium* cultures containing 2-5 % (w/v) Sucrose and Silwet-77 at concentration 0.02% (v/v). After dipping plants were kept in sealed bags for 1-2 days. Transformed plants were selected from harvested seed grown aseptically

on MS-plates containing appropriate antibiotic or directly on soil by spraying with Basta.

2.2.6. Double mutants construction

2.2.6.1. Double *bri1 ld* mutants

ld-2, and *bri1-4* were obtained from the Arabidopsis NASC stock centre and crossed to *bri1-201* and *ld-3*, *ld-2* respectively. The double mutants were found by selecting homozygous *ld* mutants as late flowering in F2 generation. The selected plants were self-pollinated and in the next generation dwarf-looking plants were identified to be homozygous for both *ld* and *bri1*. The F3 generation was used for flowering time analyses.

2.2.6.2. Double *bri1ga1, bri1 fca, bri1 FRI*, *bri1 gi* mutants

The *bri1-201* allele was used for flowering time analyses of double *bri1* to other flowering time mutants. For gene expression also double mutants between *bri1-202* and *gi-11, FRI*^{SF2}, and *fca-11* were obtained. Single *bri1-201/202* were used as pollen acceptor and were crossed to single *fca-11, FRI*^{SF2}, *gi-11*. In the F2 generation of these crosses, the late flowering plants were identified as homozygous for *fca-11* or *gi-11*. Plants that were found in the F3 generation to segregate *bri1-201* were used for flowering-time experiments. In case of the cross *FRI*^{SF2}to *bri1-201/202,* late flowering plants found in the F2 generation were genotyped with primers: FRIforw and FRIrev in order to find lines that were homozygous for *FRI*^{SF2}. The longer PCR products indicates the dominant *FRI* allele, the shorter one *fri* WS (Johanson *et al.,* 2000). Subsequently, plants heterozygous for *bri1-201/202* were identified and used for further experiments. The *bri1-201* was crossed to *ga1-3*. The *ga1-3* mutant was originally in the L*er* background, therefore it was 3 times backcrossed into the WS before final crosses. The obtained *ga1-3* WS was genotyped roughly on all chromosomes to test for the presence of WS/L*er* markers. Line that showed a high content of WS specific markers was chosen for further experiments. This line has also been confirmed with primers: FLCLerfor, FLCLerrev to be homozygous for the WS allele of *FLC.*

2.2.6.3. Double *aba2 ga1*, *aba2 cpd*, *ga1 cpd* mutants

Single *cpd*, *aba2*, and *ga1* mutants were crossed to each other in order to obtain double mutants. The *cpd* mutant is male-sterile, therefore it was used as a pollen acceptor plant. The *ga1* was used as a pollen acceptor in a cross to the *aba2* mutant and as a pollen donor when crossed to the *cpd* mutant. In the latter case, the *ga1* mutant was sprayed with 50 μM GA_3 solution to restore fertility to enable successful crossing.

The double mutants *aba2 cpd* was initially selected on MS-medium containing 6% glucose as this selects for glucose-insensitivity described for the *aba2* mutant (Cheng *et al.,* 2002). Plants heterozygous for *cpd* were found by testing the F3 generation for the presence of "cabbage"-looking plants. Since the *cpd* mutant is male sterile, the double homozygous mutants were always visually selected from the segregating population during each experiment. Similarly, the *ga1 cpd* double mutant was selected from the F3 population homozygous for *ga1*, and segregating *cpd*. Plants homozygous for *ga1* were recognized by dark-green leaves and stunted morphology. To isolate the *aba2 ga1* double mutant, the *aba2* mutant was crossed to the *ga1* mutant, and in the F2 generation, plants homozygous for *ga1* were selected. In the next generation, plants with less severe GA-deficiency compared to the *ga1* population homozygous to the single *ga1* mutant were chosen as putative double mutants. The candidate plants were tested for the absence of DNA band in the *aba2* mutant with gin11RT, gin11revRT (Cheng *et al.,* 2002). As a positive control that results in a band both in WT and in the aba2 mutant, gin1-1RT and aba23rev were applied. Identified in this way the *aba2 ga1* mutant was self-fertilized and its progeny was used in further experiments.

2.2.7. Transgenic plants construction

2.2.7.1. Construction of plants overexpressing *DWF4, NCED3, GA5*

Genomic clones of the *DWF4, GA5,*and *NCED3* were amplified with primer pairs: dwfoxl, dwfoxr; nced3oxl, nced3r; ga5oxl, ga5oxr, respectively. Purified PCR-products were separately inserted into the pDONR207 vector by means of BP reaction. The accuracy of cloned gene sequences was confirmed by sequencing.

Subsequently, the cloned *DWF4, GA5, NCED3* genes were inserted downstream of the 35S promoter into the plant-transformation pLeela vector using LR reaction. The resulting constructs were transformed into *Agrobacterium tumefaciens* GV3101 pMP90RK strain, which was used to transform Arabidopsis plants. Seeds harvested from the transformed plants were used to select for T1 generation of transgenic plants. Transgenic plants were selected on MS-medium containing PPT, or by directly spraying with Basta plants grown on soil. Plants were confirmed to harbor a transgene by genotyping with 35S-specific primers and gene-specific primer used for cloning. Plants were backcrossed to WS, and in F2 generation lines that carried one insert (as judged by scoring the resistance to PPT) were used for further experiments. Homozygous for transgene lines were used in experiments.

2.2.7.2. Construction of *LFY::LUC*+ reporter lines

The *LFY* promoter, defined as 3.7 kb region upstream of the ATG site for the *LFY* gene was amplified with PCR from the genomic DNA with 5LFYAPROM and 3LFYAPROM primers, which add *SalI, SmaI* sites. The PCR product was cloned into the corresponding sites in the pPZP221-LUC+NosT vector, upstream from the *LUC+* gene. The accuracy of cloned promoter sequences was confirmed by sequencing. Subsequently, the resulting reporter construct was transformed into *Agrobacterium tumefaciens* ABI strain, which was used to transform Arabidopsis plants. Seeds harvested from the transformed plants were used to select for T1 generation of transgenic plants. Transgenic plants were selected on MS-medium containing gentamicin and confirmed by testing for luminescence upon spraying with luciferin. Plants were backcrossed to WS, and in F2 generation lines that carried one insert (as judged by scoring the resistance to gentamicin) were used for further experiments. Homozygous lines for the transgene were used in imaging experiments.

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

The highly specific region 5UTR region of *FLC* transcript was amplified with FLC5UTRfor and FLC5UTRrev primers from cDNA prepared from *ld* mutant (expresses *FLC* to high levels). The purified PCR product was cloned into the pDONR207 vector by means of BP reaction. The accuracy of cloned sequence was confirmed by sequencing. Subsequently, the cloned *FLC* fragment was inserted in two

inverted copies into the plant-transformation pJawohl8-RNAi vector using LR reaction. The resulting constructs were transformed into *Agrobacterium tumefaciens* GV3101 pMP90RK strain, which was used to transform Arabidopsis plants. Seeds harvested from the transformed plants were used to select for T1 generation of transgenic plants. Transgenic plants were selected by directly spraying with BASTA plants grown on soil. T2 generation was used in experiment.

2.2.7.4. Construction of *35S::GA5/35S::DWF4* line harboring *LFY::LUC+*

The *35S::GA5/35S::DWF4* line harboring *LFY::LUC+* were obtained by crossing selected *35S::GA5/35S::DWF4* lines with the chosen *LFY::LUC+*. Double transgenic lines were isolated by selecting for Basta resistance and for luminescence upon spraying with luciferin. Double homozygous lines were used in experiments.

2.2.8. Plant growth conditions

For gene-expression studies and camera imaging, plants were grown on MS-medium without sucrose in the Percival growth chambers, at 22°C under the long-day photoperiod (16 hours of light/8 hours of darkness) or under the short-day photoperiod (8 hours of light/16 hours of darkness). The light intensity under long days was 126-153 µmol s^{-1} m^{-2} and under short days 130-190 µmol s^{-1} m^{-2}.

Flowering time experiments with the double *bri1* to flowering time mutants were conducted in the controlled Percival chambers. The long-day conditions consisted of 10 hours of full light with 6 hours extension supplied by incandescent bulbs and the light intensity was 170-180 µmol s^{-1} m^{-2} for the first 10 hours and 4-8 µmol s^{-1} m^{-2} during last 6 hours of the light period. The short day-condition consisted of 10 hours of light and 14 hours of darkness, the light intensity was 125-195 µmol s^{-1} m^{-2}.

Flowering-time experiments with the double hormonal mutants were performed in the greenhouse. The long day consisted of 16 hours of light, followed by 8 hours of darkness; the light intensity was 80-160 µmol s^{-1} m^{-2}. The short day-condition consisted of 8 hours of light and 16 hours of darkness, the light intensity was 100-150 µmol s^{-1} m^{-2}.

2.2.9. Flowering time analyses

Seeds were sown on MS-medium, stratified for 2-5 days at 4°C in darkness, and transferred to soil. Plants were grown under long- or short-day conditions as described above. 5 -18 plants per genotype were analyzed in each experiment. Flowering time was scored as a number of rosette leaves at flowering when the bolt was *ca*. 1 cm high.

2.2.10. Vernalization treatment

Seeds were sown on MS-medium, stratified for 2 days at 4°C in darkness, followed by 2-days-incubation at 22 °C under the 12h of light/12 h of darkness photoperiod in order to induce synchronized germination. Germinated seeds were transferred to 4°C, short-day photoperiod (8 h of light/16 h of darkness), and vernalized After 6 weeks, seedlings were transferred to separate pots and grown under the long-day photoperiod consisting of 10 hours light with 6 hours extension supplied only by incandescent bulbs.

2.2.11. Imaging with a CCD camera

Seeds were surface-sterilized, sown on MS-medium, stratified for 2 days at 4°C in darkness, and transferred to short-/long-day conditions. After 7 days, 9-12 seedlings were transferred to fresh MS-medium and sprayed with luciferin in order to inactivate accumulated luciferase, so later luminescence-measurements would mirror the transcriptional activation of the promoter of interest. Luciferin was used in excess (5 mM), in order to make sure that detected LUC activity is proportional to promoter activity. Plants were sprayed with luciferin before each measurement. Also, before imaging, plants were kept in darkness for 10-20 min to decrease the level of chlorophyll chemiluminescence. The single-photon-counting liquid-nitrogen cooled CCD camera was used to monitor emitted luminescence (Visitron Systems). Each plate was imaged 5-10 min, depending on the experiment. For imaging and data analyses MetaMorph (Universal Imaging Corporation) imaging software were used.

3. Results

3.1. Genetic and molecular genetic analyses of the *BRI1*-regulated flowering

In the genetic screen aimed to identify additional modifiers of flowering time in Arabidopsis, two alleles of *bri1* were found to enhances strongly the late flowering of the autonomous mutant *ld* (Davis, unpublished). *BRI1* encodes a leucine-rich repeats receptor-like-kinase (LRR-RLK) that function as a receptor for BRs (Wang *et al.,* 2001; Kinoshita *et al.,* 2005), thus the result of the screen indicated that *BRI1* or BRs could play a role in the floral timing. In this sub-chapter, genetic and molecular-genetic approaches were used to place *BRI1* in flowering-genetic network. Various double mutant combinations between the *bri1* alleles and known flowering-time mutants were constructed and their flowering time was analyzed. Furthermore, gene expression studies of key flowering-time genes were performed to demonstrate the molecular mechanism *BRI1*-controled of flowering time.

3.1.1. Genetic interaction between *BRI1* and *LD* is not allele specific

To exclude the possibility that the extreme late-flowering phenotype of the *bri1 ld* double mutants isolated via forward genetics of flowering time was specific to *ld-3* allele used in the screen or the *bri1* alleles isolated, the phenotype was reconstituted with alternative alleles of *bri1* and *ld.* This test allowed also excluding the presence of second-site mutations that may also be present. For this, the null *bri1-4* allele was included to confirm that modifying effect on flowering time on *ld* resulted from the loss of *BRI1* activity. An independent allele of *ld* (*ld-2*) was obtained and crossed to the *bri1-201* and *bri1-4* mutants. The null *bri1-4* allele was also combined with *ld-3* (originally used in the screen). The double homozygous lines (*ld-2 bri1-4, ld-3 bri1-4, ld-2 bri1-201*) were isolated by phenotypic identification, as described in Chapter 2.

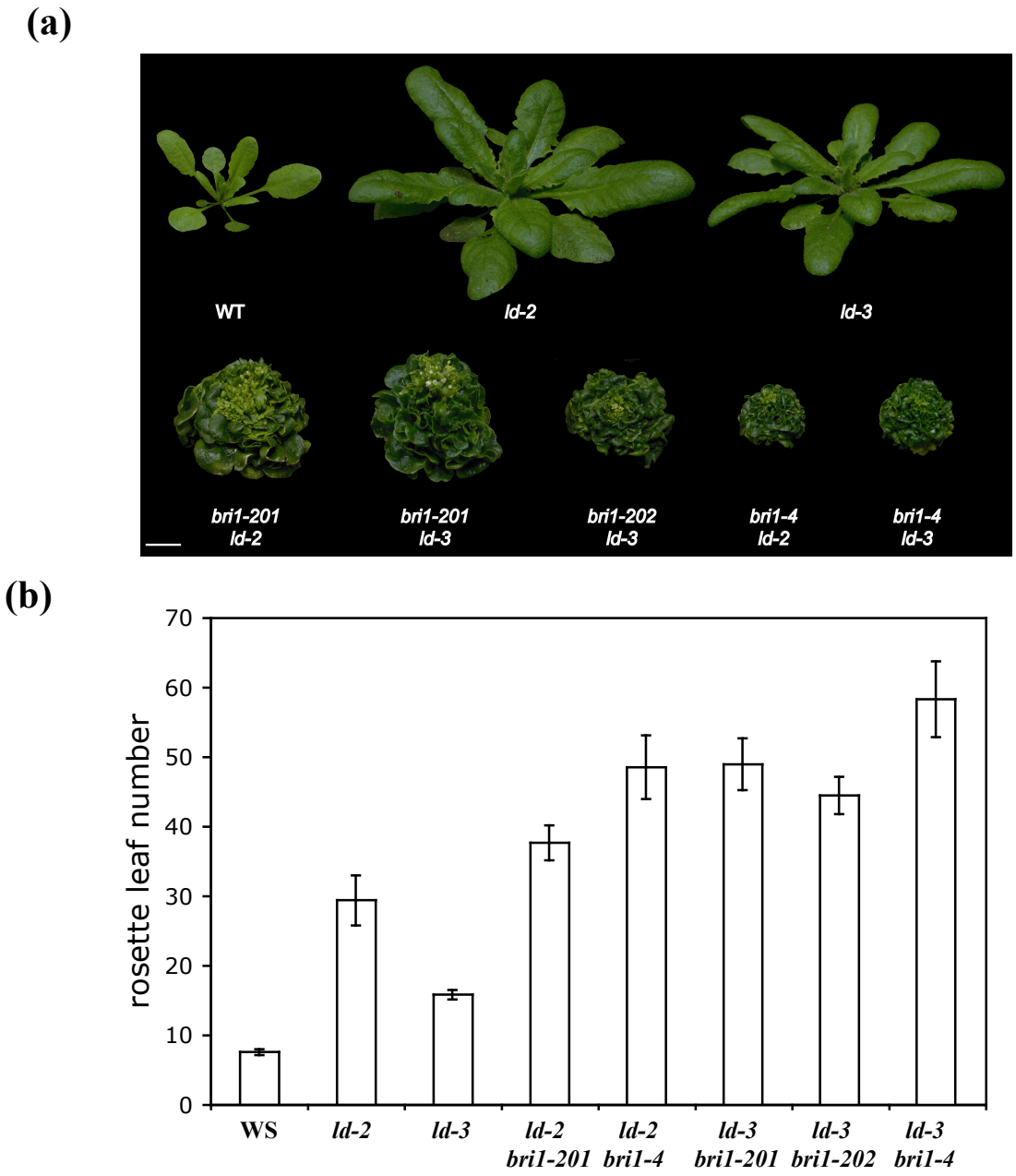

Figure 3.1. Flowering-time analyses of various *ld bri1* double mutant combinations. (a) Phenotypes of wild type, *ld-2*, *ld-3,* and various double *bri1 ld* mutant combinations: *bri1-201 ld-2*, *bri1-201 ld-3*, *bri1-202 ld-3, bri1-4 ld-2* and *bri1-4 ld-3*. Plants were grown under long days (16h light/8 h darkness) in the greenhouse; photos were taken at the time of floral inititation as assayed by inflorescence visual detection. The white bar indicates 1 cm. (b) Flowering time of the analyzed mutants. Flowering time was measured as the total number of rosette leaves formed when the bolt was *ca.* 1 cm high. Error bars represent SE. All *bri1 ld* double mutants flowered later than the respective single *ld* mutants. The null allele of *bri1* (*bri1-4*) had a stronger effect on flowering than the weak *bri1-201* allele.

The obtained double mutants were subjected to flowering time analyses under long days in the greenhouse (Fig. 3.1). The original double mutants found in the

enhancer screen were also included in this experiment. In general, all double *ld bri1* mutants flowered later than the respective single *ld* mutants. The *ld-2* allele was enhanced by both *bri1-201* and *bri1-4*, and the *bri1* null allele had a stronger modifying effect than the much weaker *bri1-201* allele. The double *ld-3 bri1-4* was the latest flowering of all double mutants included in this analysis. Altogether this indicates that loss-of-function mutations in the BR-receptor *BRI1* cause delay in flowering of the autonomous mutant *ld*, and that this effect is neither *ld*-allele specific, nor *bri1*-allele specific. Collectively, these results are confirmation of a genetic interaction between *LD* and *BRI1*.

3.1.2. *bri1* is a strong enhancer of *ld, fca* and *FRI*

To verify whether the modifying activity of *bri1* on flowering time is specific to the *ld* mutant, the autonomous pathway, or if it is a more pleiotropic effect on flowering time, the *bri1-201* mutant was combined with a different autonomous- (*fca-11*), with a photoperiod- (*gi-11*), and with a gibberellin- (*ga1-3*) pathway mutants, and a *FRI*-containing line (dominant *FRI* allele establishes a vernalization requirement), respectively. The double mutants (*fca-11 bri1-201, FRI bri1-201, gi-11 bri1-201, ga1-3 bri1-201*) were constructed by crossing the respective single mutants. The double mutants were isolated by genotyping and phenotypic identification, as described in Chapter 2. The flowering time of the resulting double mutants was measured.

Under long days, double *bri1 fca, bri1 FRI* and *bri1 ld* mutants exhibited a similar, extremely late-flowering phenotypes compared to the single *fca, FRI* and *ld* lines (Fig. 3.2, 3.3). The *gi* mutant being impaired in the photoperiod pathway, was the latest flowering single mutant under long-day condition. The double *gi bri1,* in turn, delayed flowering modestly compared to the single *gi*, and was not as late as double *fca bri1, FRI bri1* and *ld bri1* mutants. In contrast, the *ga1* mutant had under long days a mild late-flowering phenotype, but flowered later than the single *bri1*.

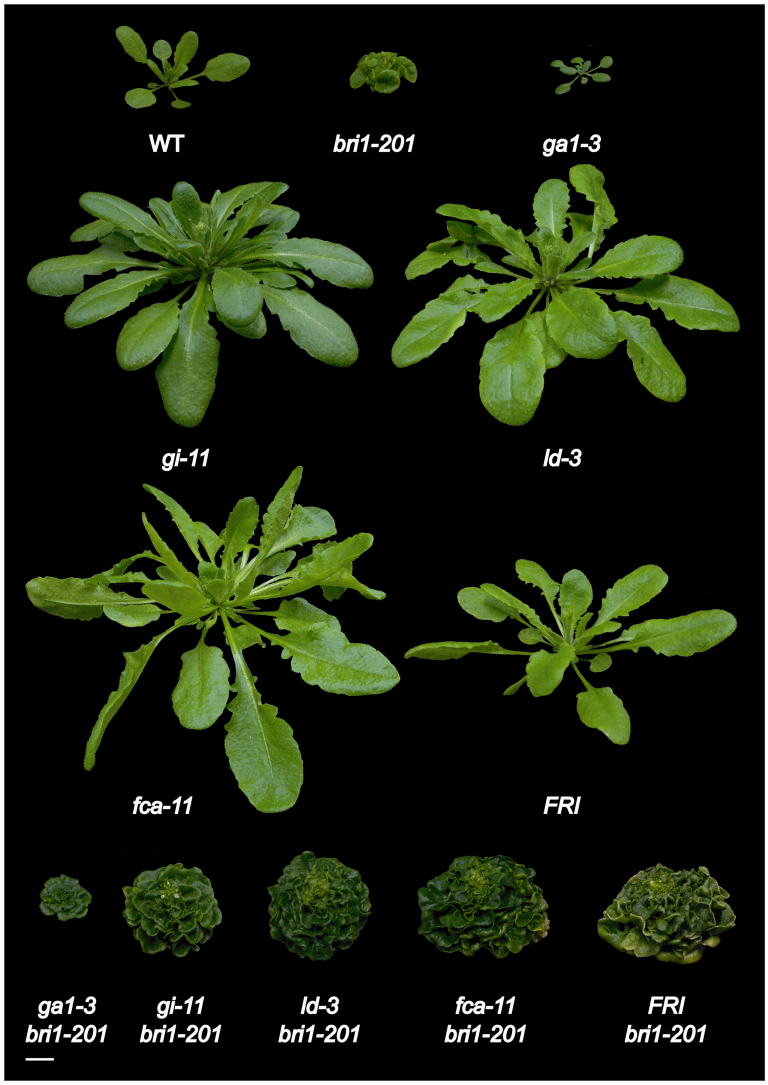

Figure 3.2. Phenotypes of the wild type WS, the single *bri1-201*, the *ga1-3*, the *gi-11*, the *ld-3*, the *fca-11*, the *FRI* lines, and the double mutants between *bri1-201* and the respective single mutants. Plants were grown in a controlled growth chamber under long days. Photographs were taken at the beginning of bolting. The white bar indicates 1 cm.

The double *ga1 bri1* exhibited later flowering than both single mutants, but was still earlier than all other analyzed double mutants with *bri1* (Fig. 3.2 and 3.3).

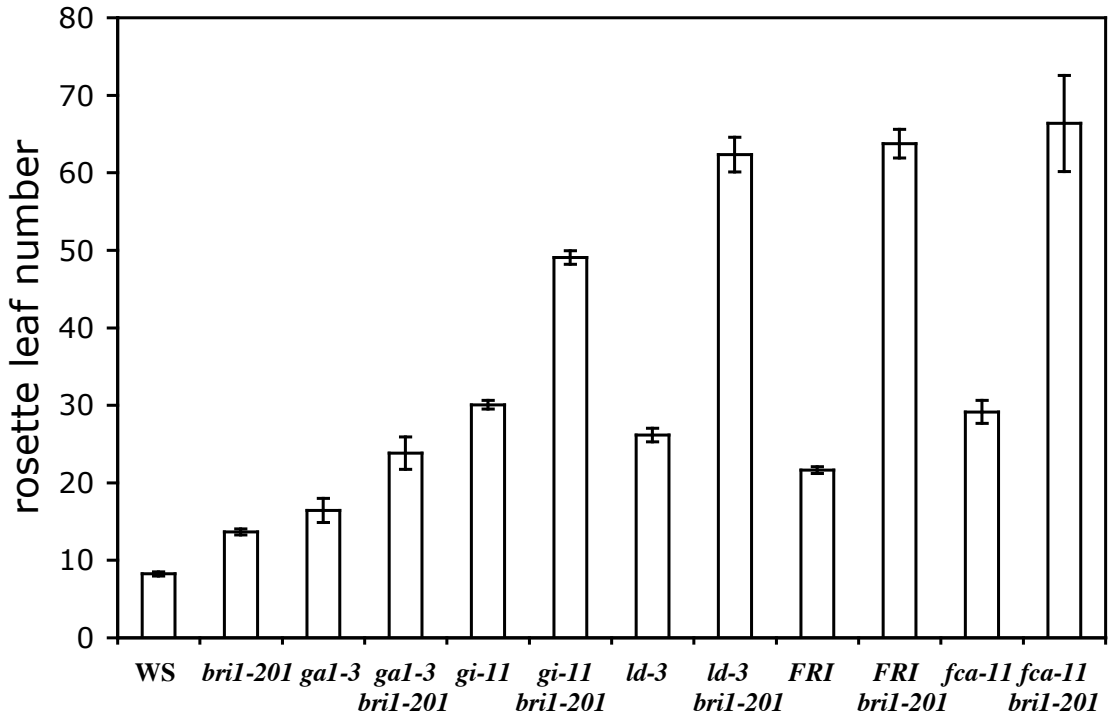

Figure 3.3. Flowering time of double mutants between the *bri1-201* mutant and different flowering time mutants: autonomous- (*fca-11*), photoperiod- (*gi-11*), gibberellin- (*ga1-3*) pathways mutants, and a *FRI*-containing line. Plants were grown in the controlled growth chamber under long days (16 h light/8h darkness) at 20 °C. Flowering time was measured as the total number of rosette leaves formed at the time of bolting. The leaf number values are averages of 10-18 plants per genotype. Error bars represent SE. Note that the *bri1 ld*/*fca*/*FRI* double mutants exhibited a similar, extremely late flowering compared to the single *ld*/*fca*/*FRI* plants. The double *bri1 gi* was also delayed compared to the single *gi* mutant, but the difference in flowering time was not as pronounced as it was for the *bri1 ld*/*fca*/*FRI* double lines. The double *bri1 ga1* mutant was delayed in an additive manner compared to the respective single mutants.

The double *bri1 ld* and the *bri1 gi* mutants were also analyzed a under non-inductive photoperiod (Fig. 3.4). Similar results as under long days were obtained; the single *bri1* mutant had a mild late-flowering phenotype and the double *bri1 ld* mutant was severely delayed in flowering compared to the single *ld* mutant. The single *gi* mutant was only slightly late flowering, because its phenotype manifests itself mainly under long days, and the double *gi bri1* mutant flowered later than both *gi* and *bri1*, but earlier than the double *bri1 ld*. These results suggest that *bri1* is additive to *ga1* and *gi,* and synergistic to *ld*, *fca*, and *FRI*. Thus, this indicates that the BRI1-pathway has limited interaction with the photoperiod and the gibberellin pathways, but it might function, at least partially, through the autonomous pathway.

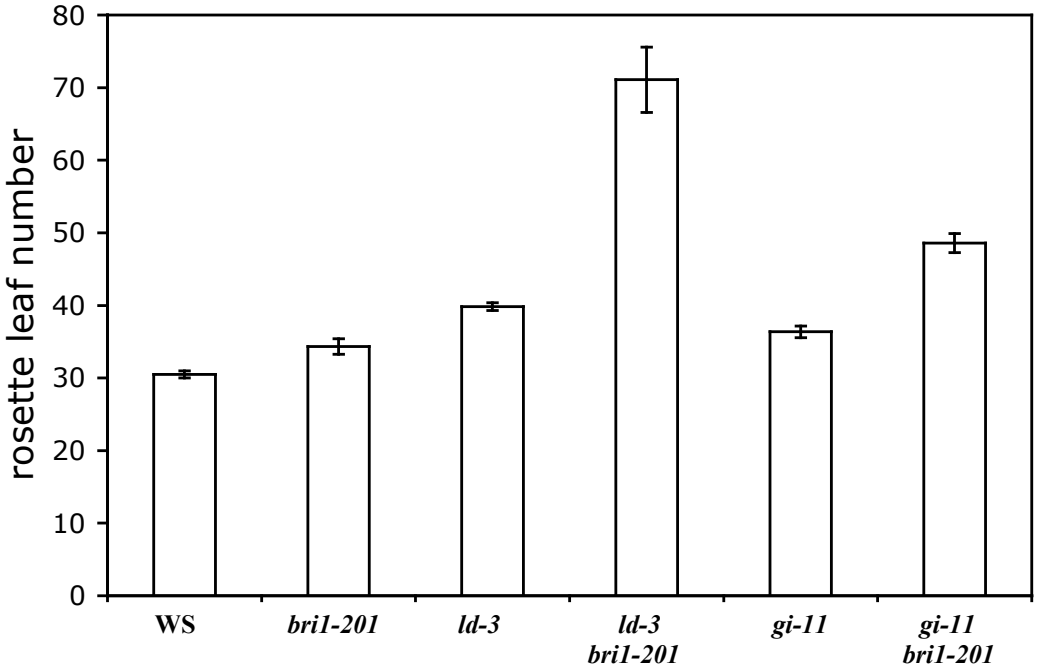

Figure 3.4. Flowering time of double mutants between the *bri1-201* mutant and the photoperiod- (*gi-11*), and the autonomous (*ld-3*) mutants under short days. Plants were grown in the controlled growth chamber short days (10 h light/14h darkness) at 20 °C. Flowering time was measured as described in Fig. 3.3. The single *bri1* mutant exhibited a modest delay in flowering. The *bri1 ld* double mutant was similarly extremely late compared to the single *ld* mutant as under long days. The single *gi* mutant had a mild late-flowering phenotype, because its phenotype is mainly apparent under the inductive photoperiod. The double *bri1 gi* flowered late in an additive manner compared to the respective single mutants.

3.1.3. *FLC* mRNA levels are higher in the *bri1 ld* mutant than in the *ld* mutant

The autonomous pathway and *FRI* regulate the expression of a potent floral repressor, *FLC*. Genes in the autonomous pathway downregulate *FLC*, while *FRI* acts as its activator. Consequently, the autonomous mutants and *FRI*-carrying lines have the same molecular phenotype, which is the accumulation of high levels of *FLC* transcript (He and Amasino, 2004). Thus, if the *BRI1*-pathway interacts with the autonomous pathway to regulate flowering, it would be expected that the double *bri1* to autonomous-pathway mutants is affected in the level of *FLC* mRNA compared to the single mutant.

To address whether *bri1* affects the levels of *FLC* in the *ld* mutant, *FLC* expression was investigated in the double *bri1-201 ld-3* mutant, and in the single *ld-3,*

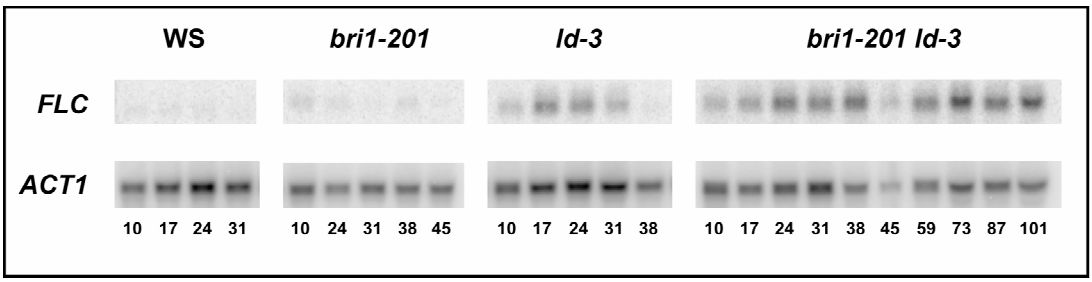

Figure 3.5. *FLC* mRNA levels in wild-type WS, the *bri1-201*, the *ld-3* and the *bri1-201 ld-3* double mutants, as monitored by RNA-blot analyses. The *ACTIN1* (*ACT1*) gene was used as a loading control. Plants were grown in a controlled environment, under long days (16 h light/8 h darkness) at 20 °C. Samples were taken at the time indicated (number of days), till the flower buds were visible. Different numbers of samples for each genotype reflect the differences in flowering time. In WS and in *bri1-201* only traces of *FLC* transcript were detected. The *ld-3* mutant contained high levels of *FLC* mRNA, which decreased with the plant age. The double *bri1-201 ld-3* contained more *FLC* transcript than the single *ld* mutant. Such high amounts of *FLC* mRNA could be still detected in the more than 3-months-old plants.

bri1-201 and WS wild type. Plants were grown under long or short-day conditions, and *FLC* levels were monitored throughout development (till flower buds were visible) using RNA-blot analysis. The results obtained for plants grown under long days are presented in Fig. 3.5. As expected, only low amounts of *FLC* were detected in the wild type, but the levels were quite high in the *ld* mutant. Interestingly, *FLC* expression seemed later in development to be repressed and finally decreased to levels as low as observed in wild-type plants. The observed decrease in the amount of *FLC* transcript correlated with the time of flowering of the *ld* mutant. The *bri1* mutant had low levels of *FLC* transcript, comparable to the levels in the wild type. In the *bri1 ld* double mutant, *FLC* transcript accumulated to much higher levels than in the single *ld* mutant. Moreover, levels of *FLC* remained very high (higher than the highest levels in the *ld* mutant) even in *ca.* 100-days-old plants. Since *FLC* is a known strong repressor of flowering, high levels of *FLC* expression in the *bri1 ld* double mutant are likely the cause of its extremely late-flowering phenotype. This result further supports the previous genetic findings that the *BRI1* pathway functions similarly to the autonomous pathway to repress the expression of *FLC.*

3.1.4. The elevated levels of *FLC* mRNA are the common feature of the double *bri1 ld*/*fca*/*FRI* plants.

The common feature of *ld*, *fca,* and *FRI*-containing lines is the high level of *FLC* mRNA. This is a cause for a similar photoperiod-sensitive late-flowering phenotype of these plants (He and Amasino, 2004). The similar flowering-phenotype of double *bri1 ld*/f*ca*/*FRI* mutants, raised the question as to whether *bri1* enhanced the *fca* mutant and *FRI*-containing line through the same molecular mechanism – increase in the level of *FLC* expression - as it does with *ld.* To address this question, *FLC* levels were examined in double *bri1 ld*/f*ca*/*FRI* mutants, and the single mutants. Plants were grown under long days and samples were taken after 30 days of growth. This experiment was performed with both alleles of *bri1*: *bri1-201, bri1-202,* and double mutant combinations with these two alleles. The wild-type plants were excluded from this experiment, because they had already flowered. After 30 days of growth, *ld bri1* and *fca bri1* double mutants with both *bri1* alleles contained higher amounts of *FLC* transcript than the single *ld* and *fca* mutants, respectively (Fig. 3.6). In case of the double *FRI bri1*, the *bri1-202 FRI* clearly contained more *FLC* mRNA compared to the single *FRI*-carrying plant, but the *bri1-201 FRI* seemed to have similar or only slightly increased levels of *FLC*. All together, this suggests that *bri1* delays flowering of the *ld/fca/FRI* lines through the same molecular mechanism, the enhancement of the expression of the strong floral repressor *FLC*.

3.15. The floral integrators are downregulated in the double *bri1 ld*/*fca*/*FRI* lines.

To further investigate the molecular mechanism of the late flowering of the double *bri1 ld/fca/FRI* plants, the levels of the floral pathways integrators - *SOC1, FT*, and *LFY* - in these lines were compared to the single *ld/fca/FRI* plants. 30-days-old plants grown under long days (as described in chapter 3.14) were used in this experiment. The levels of *SOC1*, *FT,* and *LFY* transcripts were clearly lower in the single *ld/fca/FRI* and the double *bri1 ld/fca/FRI* plants than in the single *bri1* mutants (Fig. 3.7). Moreover, *SOC1* accumulated to lower levels in the double *bri1 ld/fca/FRI* plants than in the single *ld/fca/FRI.* The lower levels of *SOC1* transcript reflect the

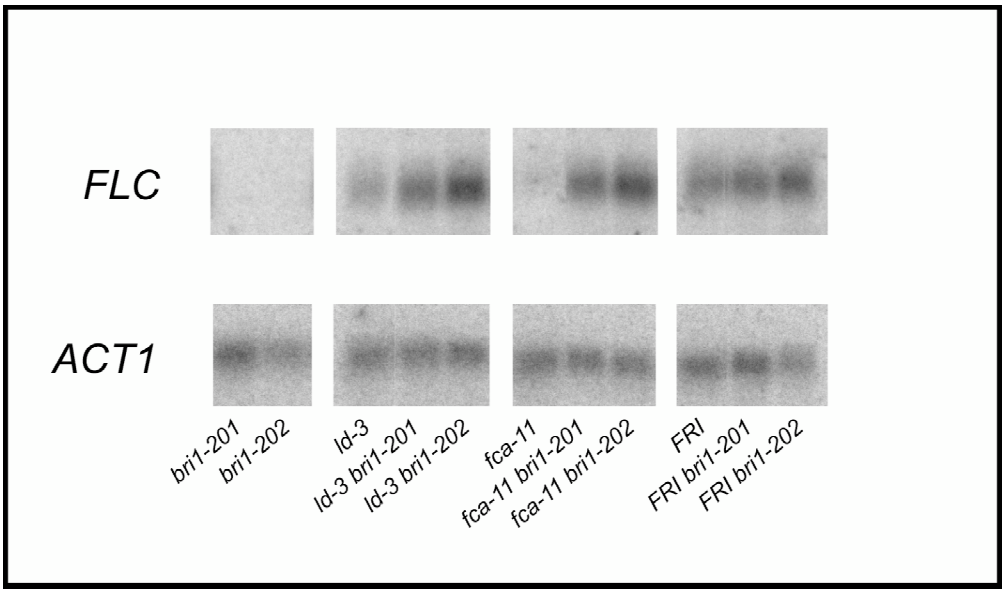

Figure 3.6. *FLC* mRNA levels in the single *bri1-201*, *bri1-202, ld-3, fca-11, FRI* and the double mutants between *bri1-201*/ *bri1-202* and the *ld-3, fca-11, FRI* lines as monitored by the RNA-blot analyses. The *ACTIN1* (*ACT1*) gene was used as a loading control. Plants were grown as described in Fig. 3.5. Samples were taken at the 30th day of growth. The *FLC* transcript was undetectable in the single *bri1* mutants. In the double *ld*/*fca bri1* mutants, *FLC* clearly accumulated to higher levels than in the single *fca* and *ld* mutants. In the double *FRI bri1* mutants, the *FLC* levels also seemed to be higher than in the single *FRI* line, however, the effect was not so evident.

higher abundance of the *FLC* mRNA in these plants, further supporting that the high *FLC* level is the main cause of the extreme late flowering of the double *bri1 ld/fca/FRI* lines. Due to sensitivity restrictions, it was impossible to distinguish whether the *FT* and *LFY* abundance is lower in the double *bri1 ld/fca/FRI* plants than in the single *ld/fca/FRI.* However, it was doubtless that the levels of these two integrators were extremely low both in the single *ld/fca/FRI* and in the double *bri1 ld/fca/FRI* plants at the tested time point (Fig. 3.7). In conclusion, the double *bri1 ld/fca/FRI* lines contain remarkably low levels of three known floral pathways integrators, thus, reduced expression of floral-pathway integrators caused by elevated levels of *FLC* constitute the molecular basis underlying the severe delay in flowering of these plants.

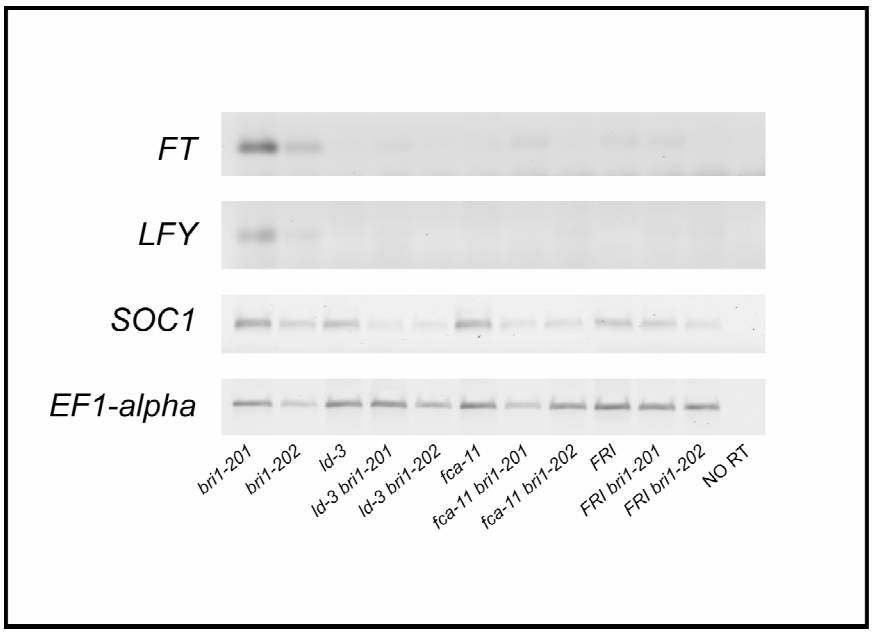

Figure 3.7. Expression of the floral pathway integrators: *FT, LFY, SOC1* in the single *bri1-201*, *bri1-202, ld-3, fca-11, FRI* and the double mutants between *bri1-201*/ *bri1-202* and the *ld-3, fca-11, FRI* lines, as examined by RT-PCR. Primers specific for the elongation factor 1-alpha gene were used as a control. The *FT* and *LFY* transcripts could only be detected in the single *bri1* mutants. The *SOC1* levels were decreased in the double *ld*/*fca bri1* mutants compared to the single *ld*/*fca* mutants, while in the double *FRI bri1* mutants compared to the single *FRI* line, *SOC1* might be only slightly downregulated. The *SOC1* levels correspond to the levels of *FLC* mRNA detected in these plants.

3.16. Vernalization suppresses late flowering of the double *bri1 ld/fca/FRI* lines

Vernalization treatment is a well-described process that promotes flowering. In particular, the late-flowering phenotype of plants that contain high-levels of *FLC* can be almost fully suppressed by a prolonged exposure to cold (Sung and Amasino, 2004). Therefore, if the main cause of the extremely late flowering of *bri1 ld*/f*ca*/*FRI* double mutants is the high level of *FLC* transcript, double mutants between *bri1* and autonomous mutants or *FRI*-carrying line should have a comparably early-flowering phenotype as the respective single mutant upon the exposure to low temperatures. Importantly, acceleration of flowering by the vernalization treatment would only occur if the *BRI1*-pathway were separate from the vernalization pathway.

To investigate whether the BRI1 pathway is distinct from the vernalization pathway, double *bri1* to *ld*/*fca*/*FRI* mutants, single mutants and wild type were subjected to a vernalization treatment. Plants were grown under a short photoperiod at

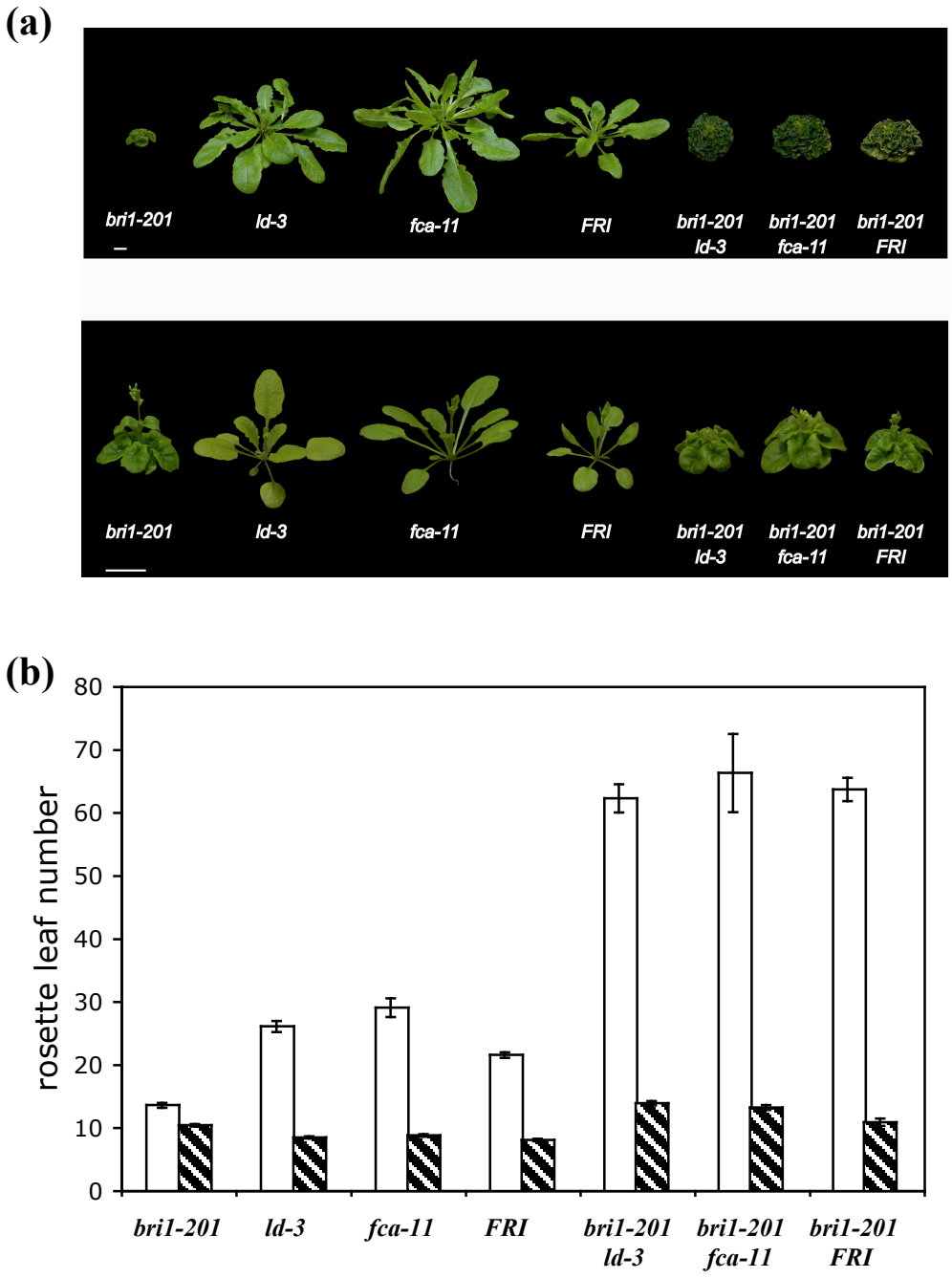

Figure 3.8. Flowering-time analyses of plants exposed to vernalization treatment in comparison to non-vernalized plants. (a) Phenotypes of the non-vernalized (-V) and vernalized (+V) *bri1-201, ld-3*, *fca-11, FRI* and the double *bri1-201 ld-3*/*fca-11*/*FRI* double mutants. Non-vernalized plants were grown as described in Fig 3.4. Vernalized plants were grown for 6 weeks under short days (8 h light/16 h darkness) at 4 ˚C, after which they were grown in the same conditions as non-vernalized plants. The pictures were taken at the beginning of flowering. The white bars on each figure indicate 1 cm. (b) Flowering time of the analyzed lines. The open columns represent non-vernalized controls, the dashed columns represent the vernalization treatments. Flowering time was scored as described in Fig. 3.4. All plants analyzed in this experiment accelerated flowering in response to vernalization.

4°C for 6 weeks, after which they were moved to the long-day condition and their flowering time was scored. All genotypes tested in the vernalization experiment strongly responded to the vernalization treatment by accelerating flowering (Fig. 3.8). The double *bri1 ld*/f*ca*/*FRI* mutants flowered almost at the same time (as scored by the rosette leaf number) as single *ld*/f*ca*/*FRI.* This indicates that *bri1* does not interact with the vernalization pathway and further supports that *BRI1* functions as a modulator of the autonomous pathway

3.17. The reduction of *FLC* levels via RNAi strongly accelerates flowering of the *bri1 ld* double mutant

If *FLC* is the major factor determining late flowering of the double *bri1 ld* mutant, a reduction in of *FLC* function should suppress the late flowering phenotype of the double mutant. Since a loss-of-function allele of *flc* in WS was not available, RNA interference (RNAi) technology was applied to reduce the levels of *FLC* mRNA in the *bri1 ld* double mutant.

To avoid cosuppression of the whole *FLC*-clade, a highly specific 5'UTR fragment of *FLC* transcript was used for silencing. The PCR-amplified fragment was cloned into pJawohl8-RNAi, a binary plant gene-silencing vector. The vector used in this experiment enables insertion of one fragment in two copies, which are inverted and joined with an intron. Upon stable transformation in plants, the constitutively expressed transgene forms a self-complementary hairpin RNA (hpRNA), which mimics double-stranded DNA (dsDNA) and provides specificity of the RNAi. The construction of the FLC-RNAi vector and obtaining transgenic plants carrying the vector is described in detail in Chapter 2.

To analyze the effect of the reduction of the *FLC* transcript in the *bri1 ld* double mutants, 10 lines transformed with the silencing construct and two lines transformed with the control plasmid were chosen for flowering-time measurements. The experiment was carried out in the T2 generation, therefore plants harboring the transgene were first selected based on their resistance to herbicide phosphinothricin. The flowering-time experiment was performed in the greenhouse, under long-day

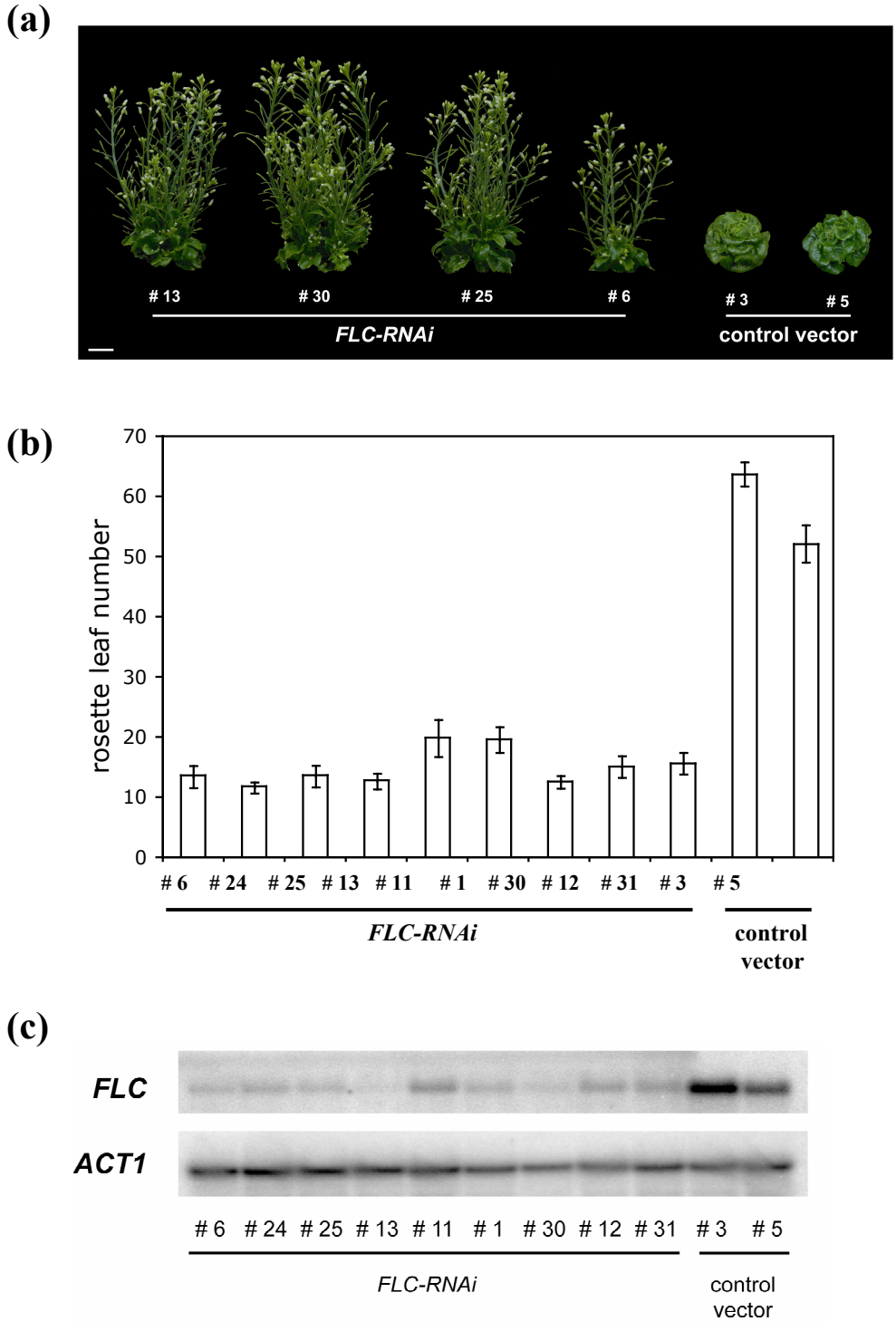

Figure 3.9. Analyses of the effect of *FLC-RNAi* on flowering time in the *bri1-201 ld-3* background. (a) Phenotypes of four representative *FLC-RNAi* in the double *bri1-201 ld-3* mutant background. As a control, two double *bri1-201 ld-3* mutants transformed with a control vector are shown. 7-week-old plants are shown. Plants were grown as described in Fig. 3. 1. The white bar indicates 1 cm. (b) Flowering-time analyses. Flowering time was measure as described previously. All *FLC-RNAi bri1 ld* lines flowered much earlier than the control *bri1 ld* plants carrying the control vector. (c) RNA-blot analyses of the *FLC* levels. The *FLC-RNAi* construct markedly reduces the amount of the *FLC* mRNA. The reduction in *FLC* abundance correlates with the strong acceleration of flowering.

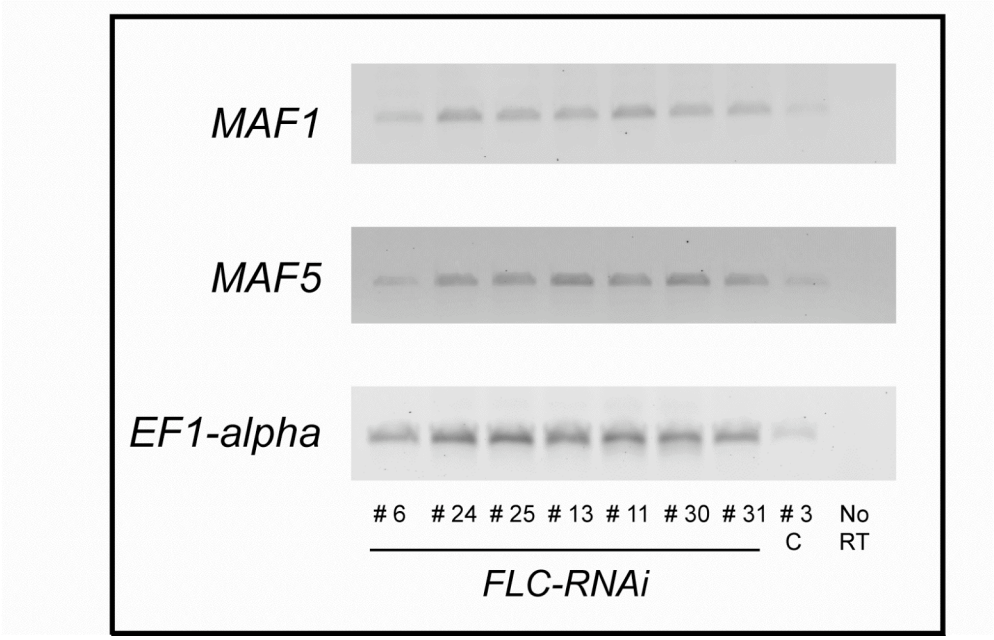

Figure 3.10. The levels of two *FLC*-related genes: *MAF1* and *MAF5* in the *FLC-RNAi bri1-201 ld-3* lines. Gene expression was measured using RT-PCR with gene-specific primers. *EF1*-alpha was used as a control. c – the control *bri1-201 ld-3* plant transformed with the control vector. No RT is a control for DNA contamination in RNA samples. No apparent decrease in the *MAF1* and *MAF5* levels was observed in the *FLC-RNAi* lines compared to the control non-silenced plant.

conditions (Fig. 3.9 a, b). Moreover, RNA-blot analysis was carried out to confirm the reduction in *FLC* mRNA in transgenic plants (Fig. 3.9 c). All plants harboring the *FLC-RNAi* transgene showed accelerated flowering compared to plants carrying the control vector (Fig. 3.9). Also, these plants were found to have significantly lower levels of *FLC* transcript, compared to the non-silenced plants. Furthermore, to test the specificity of the silencing, mRNA levels of two *FLC*-relatives: *MAF1* and *MAF5* were analyzed in the FLC-RNAi lines. Semi-quantitative Reverse Transcription PCR (RT-PCR) with gene-specific primers for each gene was applied to assess the abundance of *MAF1* and *MAF5* transcripts. As a control, RT-PCR with *EF1*-□-specific primers was performed. No apparent decrease in the levels of *MAF1* and *MAF5* transcripts was detected in the *FLC-RNAi* lines compared to the control plants carrying the empty vector (Fig. 3.10). This implies that the *FLC-RNAi* construct used in this experiment specifically targets *FLC* mRNA and does not seem to silence the *FLC*-related, *MAF*-family genes. In conclusion, the *FLC-RNAi* experiments in the

double *bri1 ld* mutant confirms that the level of *FLC* plays a major role in delaying the flowering-time of this double mutant.

3.18. Discussion

As a result of an enhancer genetic screen, two independent alleles of *bri1* were isolated as strong enhancers of the late-flowering phenotype of the autonomous mutant *luminidependens* (*ld*) (Davis, unpublished). *BRI1* encodes an LRR-RLK that functions as a receptor for BRs, thus this suggested that *BRI1* could be involved in the regulation of floral timing. In this chapter, the role of *BRI1* in the control of the transition to the reproductive growth was studied. Genetic and molecular-genetic approaches were applied to place *BRI1* in a flowering-genetic network. Various double mutant combinations between the *bri1* alleles and known flowering-time mutants were constructed and their flowering time was analyzed. Furthermore, gene-expression studies of key flowering-time genes were performed to demonstrate the molecular mechanism through which *BRI1* controls flowering time.

At first, the enhancing activity of *bri1* on the *ld* mutant was confirmed with the null allele of *bri1* combined with an alternative allele of *ld* (Fig. 3.1). All double *bri1 ld* allele combinations led to an enhanced late-flowering phenotype compared to the single *ld* mutant. This experiment allowed the exclusion of any allele-specific effect of *ld,* and thus, the presence of any additional mutations in the original *ld* mutant used for the screen. Moreover, it could be concluded that *BRI1* and *LD* genetically interact to regulate flowering time.

Next, the specificity of the effect of *bri1* on flowering time was tested by combining *bri1* with known flowering-time mutants, which have been previously placed in different genetic pathways. These included: another autonomous mutant *fca*, a photoperiod-pathway mutant *gi*, a gibberellin-deficient mutant *ga1*, and a *FRI*-carrying line, which confers a vernalization requirement. The resulting double mutants were analysed for their flowering time, and based on this BRI1 was placed in the flowering-genetic network (Fig. 3.3). The *bri1 ga1,* and *bri1 gi* double mutants exhibited a modest late-flowering phenotype compared to the respective single

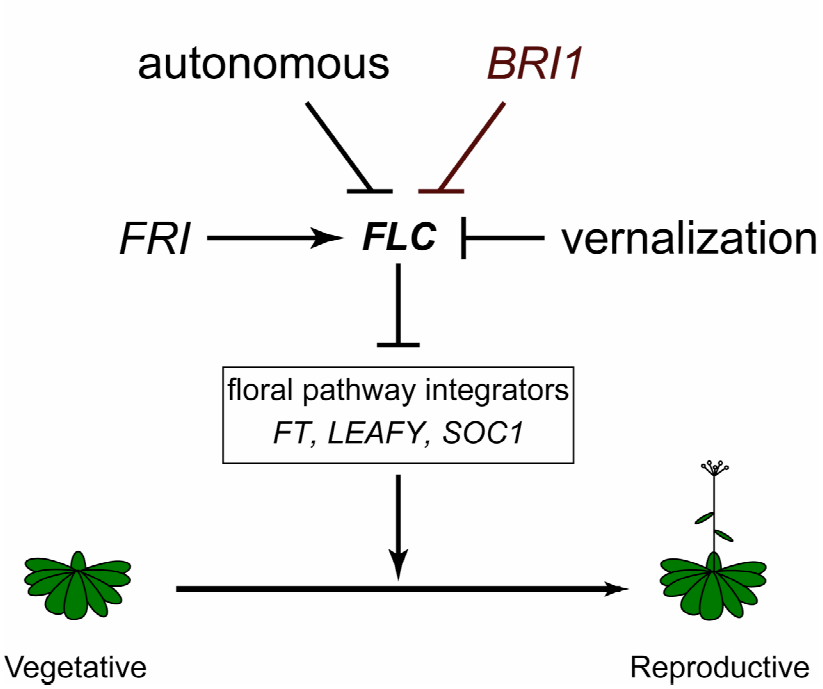

Figure 3.11. A proposed model describing the role of *BRI1* in the control of flowering time in Arabidopsis. The model was constructed based on genetic analyses of various double mutant combinations between *bri1* and known flowering time mutants. Genetic studies were supported by molecular analyses of the expression of the key flowering-time genes in the tested mutants. *FLC* seems to be the key-downstream target of the *BRI1* pathway. *BRI1* probably functions independently of the vernalization pathway, and in parallel to the autonomous pathway to repress *FLC* expression. Thus the *BRI1* pathway does not act directly to promote flowering, but rather assists in the establishment of the competence for flowering.

mutants, therefore it was concluded the *BRI1* pathway has only a limited interaction with the photoperiod and gibberellin pathways. Surprisingly, *bri1* was found to lead to extremely late flowering when combined with *FRI* and *fca* lines in a similar manner as it was observed for *bri1 ld*. Therefore, it had been proposed that BRI1 interacts with the autonomous pathway. *bri1 ld* was also found to be markedly late-flowering under non-inductive conditions, which indicates that its modifying effect on the autonomous mutant *ld* does not depend on the photoperiod (Fig. 3.4).

Since the autonomous mutants and *FRI*-carrying lines are late flowering due to the same molecular mechanism (high expression of the floral repressor *FLC*), the comparably late flowering phenotype of the *bri1 ld/fca/FRI* double mutants suggested that *BRI1* functions in parallel to the autonomous and *FRI* pathways to repress the expression of *FLC*. Thus, the levels of *FLC* expression throughout the development

were compared in the single *ld,* and *bri1* mutants to the *bri1 ld* double mutant. *bri1* was not found to have high levels of *FLC*, which is consistent with its modestly late flowering. *bri1 ld*, however, exhibited a two to three times higher expression of *FLC* than *ld* , and such high expression could still be detected in more than 3 months old *bri1 ld* plants. This indicates the severely late-flowering phenotype of the *bri1 ld* double mutant is likely due to the elevated levels of *FLC* (Fig. 3.5). To test whether *bri1* enhances *FRI* and *fca* through the same molecular mechanism, *FLC* expression was tested in double *bri1 fca,* and *bri1 FRI* mutants, with two independent *bri1* alleles compared to single *fca* and *FRI.* Double *FRI/fca bri1* mutants were found to have higher levels of *FLC* than single *FRI* and *fca,* thus confirming that *bri1* delays flowering of plants that contain high *FLC* levels by further increasing the expression of this floral repressor (Fig. 3.6).

To confirm that elevated *FLC* expression causes the observed delay in flowering of *bri1 ld/fca/FRI* double mutants, the expression of downstream targets of *FLC* was examined compared to single *ld/fca/FRI* (Fig. 3.7). The increased *FLC* expression in *bri1 ld/fca/FRI* double mutants correlated with a reduced expression of *SOC1,* and levels of *FT* and *LFY* were found to be extremely low in both single *ld/fca/FRI* and *bri1 ld/fca/FRI*. Thus, the high expression of FLC in *bri1 ld/fca/FRI* double mutants causes a reduction in the expression of floral pathways regulators, leading to their severely late-flowering phenotype.

The *bri1 ld/fca/FRI* double mutants were also subjected to vernalization, because the late-flowering phenotype of plants that contain high-levels of *FLC* can be suppressed by this treatment (Fig. 3.8). The *bri1 fca/FRI/ld* double mutants responded to vernalization by early flowering. The ability to respond to vernalization also implies that the *BRI1*-pathway is independent from the vernalization pathway.

The final experiment to confirm the major role of *FLC* levels in determining late flowering of the *bri1 ld* mutant was testing whether reduction of *FLC* levels accelerates flowering of this double mutant (Fig. 3.9). Reducing *FLC* levels via RNAi hastened flowering of the *bri1 ld* double mutant efficiently, confirming that *FLC* is the major factor regulating the flowering time of this double mutant.

The results presented here provide evidence that *BRI1* establishes a new genetic pathway that regulates the timing of floral transition in Arabidopsis. Based on

the experiments described in this chapter, a model that places *BRI1* in the genetic flowering-regulating network was proposed (Figure 3.11). In this model, *BRI1* interacts with the autonomous pathway to repress the expression of *FL,C* and it does so independently from the vernalization pathway. Given that the *bri1* single mutant has only a modest late-flowering phenotype while the autonomous mutants or *FRI* plants have much more pronounced phenotypes, *BRI1* probably has an assisting role to the autonomous pathway in the repression of *FLC*. This also implies that the *BRI1*-pathway does not function to directly promote flowering, but by enabling the repression of a strong floral repressor, which introduces the competence in the SAM to respond to floral inductive signals such as the photoperiod. Future molecular and biochemical studies will reveal what the mechanism of *BRI1* activity on *FLC* expression is.

3.2. Comparing the flowering phenotypes of a BR-receptor mutant and a BR-deficient mutant

The fact that mutations in the BR-receptor alters flowering time and genetically interact with the autonomous mutants raised the question whether this effect is due to a pleiotropic phenotype of *bri1* mutant or is due to BR deficiency.

3.2.1. The *bri1* mutant is a stronger enhancer of flowering than the *cpd* mutant

To test whether the enhancing activity of late flowering of *bri1* mutants is caused by pleiotropic effects of the mutation itself or is specific to the BR-regulated physiological processes, the effect of the reduction in endogenous BRs on flowering time was tested. The severely BR-deficient mutant, *constitutive photomorphogenesis and dwarfism* (*cpd*) was chosen for these studies. The *cpd* mutant is an extreme dwarf resulting from a block of one of the last steps of the BR-biosynthesis (Szekeres *et al.,* 1996). The severity of the phenotype of *cpd* loss-of-function mutants is comparable to the phenotype of strong alleles of *bri1* (Vert *et al.,* 2005).

The *cpd-10* mutant was introduced to the *ld-3* and *ga1-3* mutant backgrounds and flowering time of the resulting double mutants was measured and compared with the flowering time of the *bri1-201 ld-3* and the *bri1-201 ga1-3* mutants, respectively. The experiment was carried out under long-day conditions (16 h light/8 h darkness) at 20 °C. The single *cpd* mutant was slightly delayed in flowering in a similar manner as the single *bri1* mutant (Fig 3.12). Also, the *cpd ld* double mutants flowered later than the single *ld* mutant. However, the degree of the enhancement of the late flowering phenotype of the *ld* mutant by the *cpd* mutant is lower than it is for the *bri1* mutant. Interestingly, *cpd ld* and *bri1 ld* had similar bolting time. *cpd ga1* flowered slightly later than the single *ga1* (Fig. 3.13), but the difference in flowering time was not significant as tested by the Student's t-test (Table 3.1). This t-test was also used to verify the significance of late flowering of all single mutants: *bri1, cpd, ga1* compared to the wild-type plants, and to test the differences between the double mutants *ga1 bri1/cpd* compared to the respective single mutants. Except for *cpd ga1,* all lines were significantly different from the genotypes they were compared (Table 3.1).

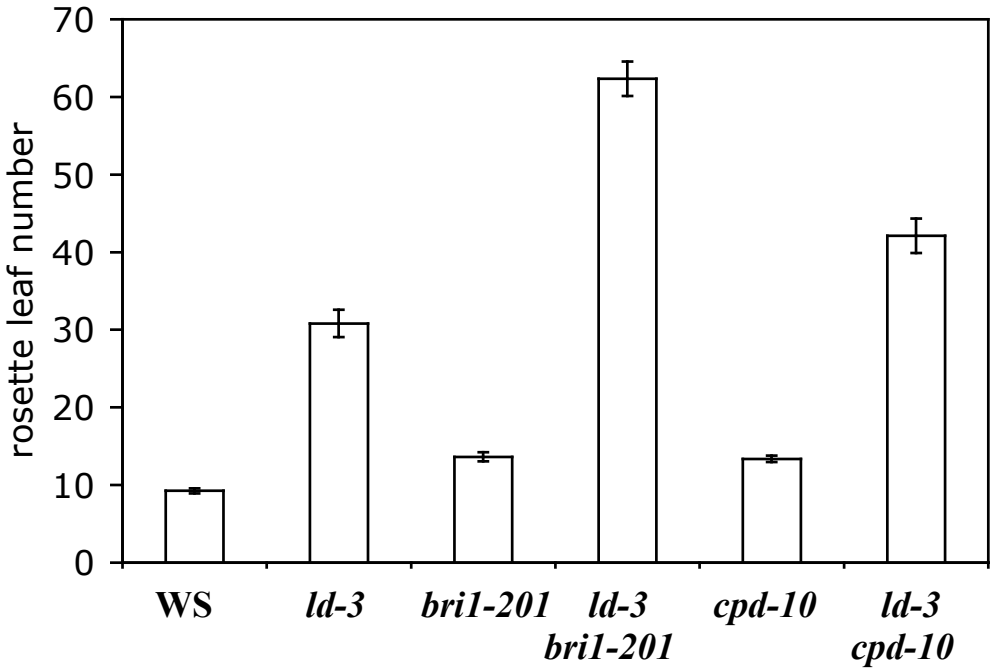

Figure 3.12. Flowering time of the wild-type WS, the single *ld-3, bri1-201, cpd-10,* and the double *ld-3 bri1-201, ld-3 cpd-10.* Plants were grown under long days (16 h light/8h darkness) at 20 °C. Flowering time was scored as the total rosette leaf number when the bolt was ca. 1 cm high. Error bars represent SE. The single *cpd* mutant exhibited a similar response to the *bri1* mutant, as it was only modestly late flowering. However, the BR-receptor mutant *bri1* enhances the autonomous mutant *ld* stronger than the *cpd* mutant does.

In particular, the double *bri1 ga1* flowered later than the *ga1* mutant and this difference was significant ($P<0.05$). Thus, the modifying activity of the *cpd* mutation on flowering time on two known flowering-time mutants: *ld* and *ga1* is weaker than the effect of the *bri1* mutantion. Perhaps, the enhancing effect of *bri1* on flowering time, is partly due to a BR-deficient response, but it is not exclusively due to such a block in BR-signal transduction.

3.2.2. The *cpd ld* double mutant accumulates higher amounts of *FLC* transcript than the single *ld* mutant

The *bri1* mutation delays flowering of the autonomous mutant *ld*, most likely by increasing the level of *FLC* transcript, thus it raised the question as to whether *cpd* has the same effect on *FLC* levels when introduced into the *ld* mutant. To address this

Table 3.1. Student's t-test for the flowering time of the single *bri1-201, cpd-10, ga1-3* mutants and the *bri1-201/ga1-3* double mutants.

Genotype 1	Genotype 2	P value
WS	*bri1-201*	0.000993
WS	*ga1-3*	0.008018
WS	*bri1-201 ga1-3*	0.000181
bri1-201	*bri1-201 ga1-3*	0.002542
ga1-3	*bri1-201 ga1-3*	0.012322
WS	*cpd-10*	0.000030
WS	*ga1-3*	0.015194
WS	*cpd-10 ga1-3*	0.000000
ga1-3	*cpd-10 ga1-3*	0.639627
cpd-10	*cpd-10 ga1-3*	0.000053

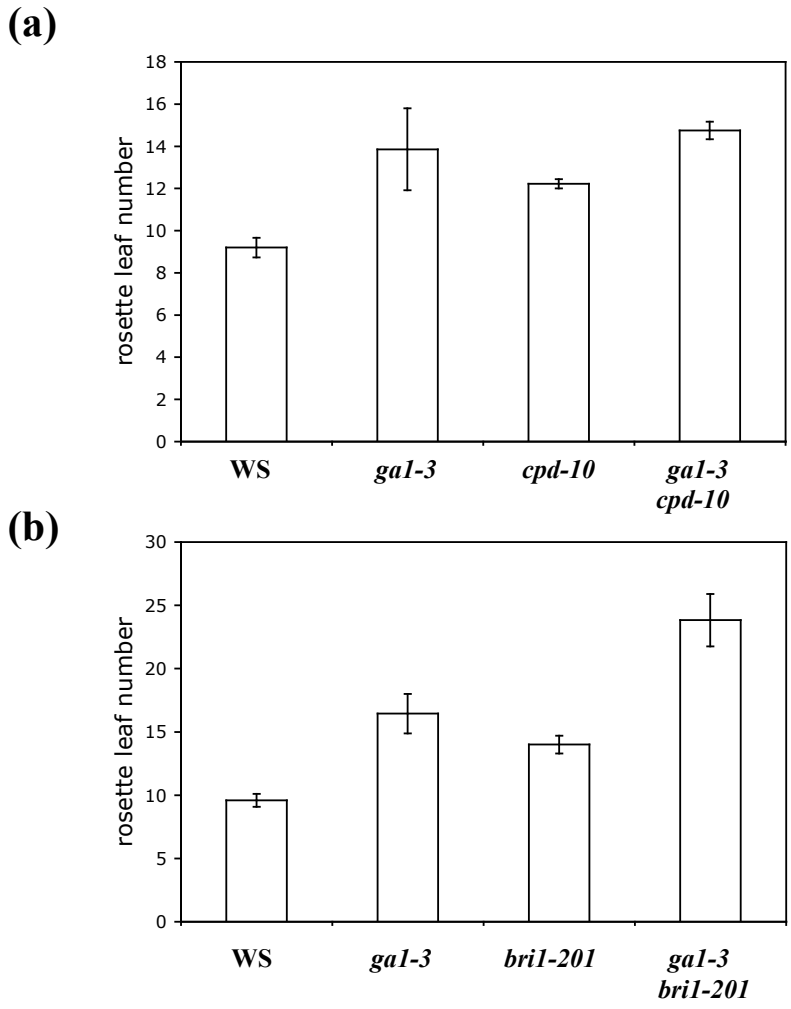

Figure 3.13. Comparing the effects of the *cpd-10* mutant (a) and the *bri1-201* mutant (b) on the flowering time of the *ga1-3* mutant. Flowering time was measured, and growth conditions were as described, on figure 3.2.1. Two experiments were performed, and representative results are shown. The flowering phenotype of the double *cpd ga1* was not significantly different from the *ga1* single mutant, while the double *bri1 ga1* flowered significantly later than the single *ga1* mutant. The differences in flowering time among the mutants were analyzed using the Student's t-test. The results of the analyses are presented in Table 3.1.

question, the level of *FLC* mRNA was examined in the *cpd-10 ld-3* double mutants compared to the single mutants and to the *bri1-201/bri1-201 ld-3* double mutants. Plants were grown under long days (16 h light/8 h darkness) at 20 °C, and the accumulation of *FLC* transcript was examined after 30 and 60 days of growth. On day 30, no *FLC* mRNA was detected in the single *cpd-10, bri1-201, bri1-202* mutants, and *cpd-10 ld-3, bri1-201 ld-3*, *bri1-202 ld-3* contained more *FLC* mRNA than the *ld* single mutant (Fig. 3.14). After 60 days of growth, samples were taken only from the double mutants, because all single mutants had already flowered by that time. On day 60, the level of *FLC* transcript further increased in *cpd-10 ld-3, bri1-201 ld-3*, *bri1-202 ld-3* compared to the levels observed on day 30 (Fig. 3.14). This could mean that *cpd* enhances the *ld* mutant in a similar manner as the *bri1* mutant. However, since the *cpd ld* double mutant grows slower than *bri1 ld* and ld mutants, the observed increase in the *FLC* levels could also reflect a delay in development in the double *cpd ld* compared to the *ld* mutant.

3.2.3. Vernalization treatment accelerates flowering more effectively in the *cpd* mutant than in the *bri1* mutant

To further characterize the flowering-time phenotypes of the BR-deficient mutant *cpd-10* and the BR-receptor mutant *bri1-201*, their response to vernalization treatment was investigated. For the cold treatment, germinated seeds were grown under a short photoperiod at 4 °C, for 6 weeks, after which they were moved to the long-day conditions and their flowering time was measured. The differences in flowering time under long days between non-vernalized and vernalized *cpd* and *bri1* were compared (Fig. 3.15). Non-vernalized *cpd* and *bri1* mutants had a similar flowering phenotype (13.36±0.41, and 13.56±0.24 rosette leaves, respectively), while after vernalization, the *cpd* mutant flowered earlier than the *bri1* mutant (8.50±0.17 compared to 10.50±0.17) (Fig 3.15). The significance of the differences in flowering time between the *cpd* and *bri1* mutants was tested using the Student'ts t-test. There was no significant difference for non-vernalized plants, but after cold treatment, the *cpd* mutant flowered significantly earlier than the *bri1* mutant ($P<0.0001$). Thus, even

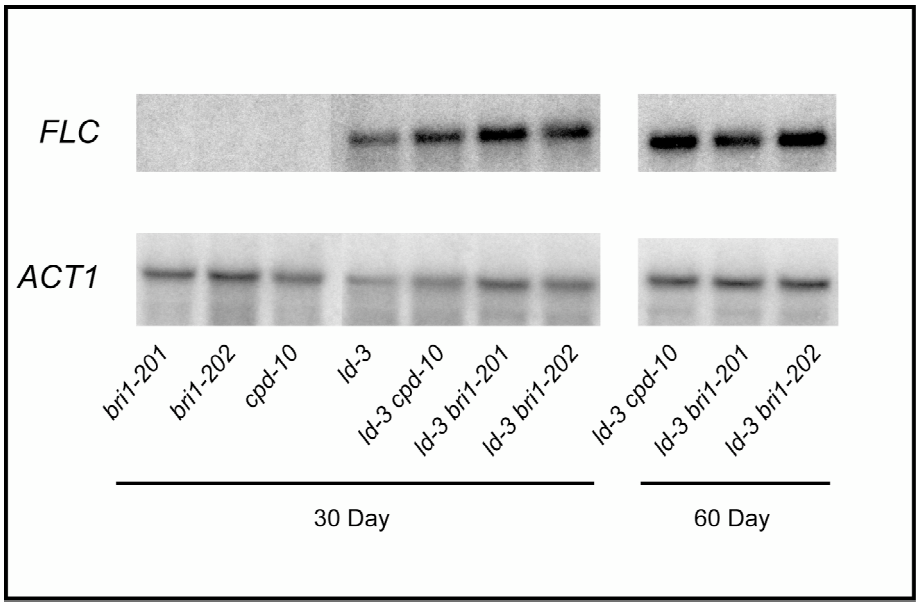

Figure 3.14. The *FLC* mRNA levels in the single *bri1-201*, *bri1-202, cpd-10, ld-3* and the double mutants between *ld-3* and *bri1-201*/ *bri1-202/cpd-10,* as monitored by RNA-blot analysis. The *ACTIN1* (*ACT1*) gene was used as a loading control. Plants were grown under long days (16h light/8h darkness) at 20 °C. Samples were taken on the 30th and 60th day of growth (on day 60 only the double mutants were examined, because the single mutants had already flowered by this time). On day 30, the *FLC* transcript was undetectable in the single *bri1* and *cpd* mutants. In the double *ld cpd* mutant, *FLC* accumulated to higher levels than in the single *ld* mutant, similar to the situation in the *ld bri1* mutants. On day 60, *FLC* levels further increased in the *ld cpd/bri1* double mutants.

though these two mutants respond to vernalization, the degree of the response is different, indicating that the nature of the late flowering of the BR-deficient mutant and BR-receptor mutant, differs at least partially.

3.2.4. Discussion

Identification of the *bri1* mutant as a strong enhancer of the late-flowering phenotype of autonomous mutants and *FRI*-carrying lines, and further molecular and genetic analyses (described in Chapter 3.1), established the *BRI1*-pathway as a genetic pathway that interacts with the autonomous pathway to control floral transition in Arabidopsis. However, it still remained unclear whether this pathway defines in fact "the brassinosteroid pathway" or additional BR-independent, but *BRI1*-dependent

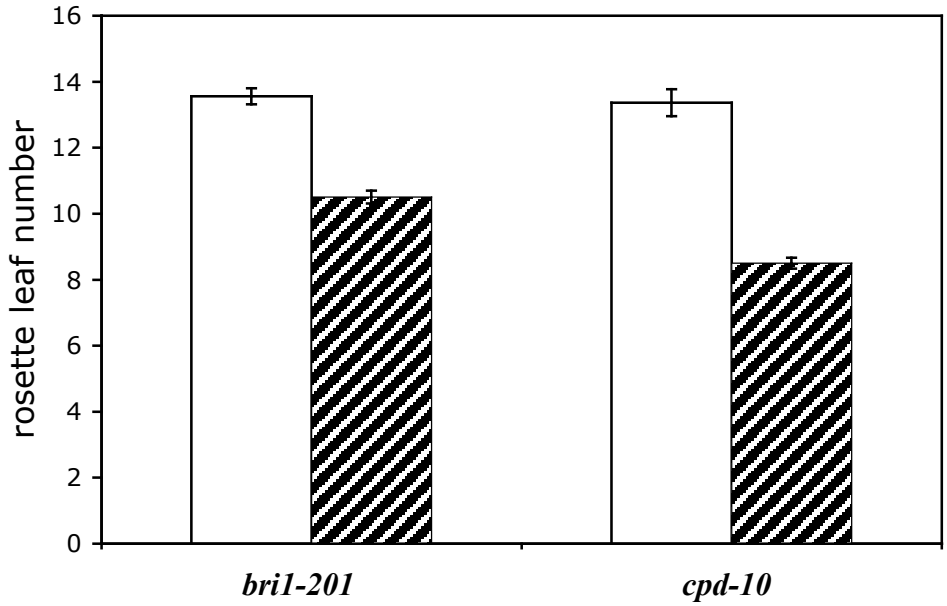

Figure 3.15. Comparison of flowering time of the *bri1-201* and the *cpd-10* mutants subjected to vernalization. Vernalized plants were grown for 6 weeks under short days (8 h light/ 16 h darkness) at 4 °C, after which they were grown in the same conditions as non-vernalized plants: under long days (16 h light/8h darkness) at 20 °C. Flowering time was scored as a total rosette leaf number when the bolt was ca. 1 cm high. Error bars represent SE. The open columns represent non-vernalized controls, the dashed columns represent the vernalization treatment. Non vernalized *bri1* and *cpd* mutants flowered with similar number of rosette leaves, while after cold exposure the *cpd* mutant flowered significantly earlier tan the *bri1* mutant ($P<0.0001$).

factors that are responsible for the described flowering phenotypes. The specificity of the *bri1* effects to BRs on flowering time was tested in this chapter.

A potential pleiotropic *BRI1* effect was addressed by comparing the flowering times of a BR-deficient mutant, *cpd* and the BR-receptor mutant *bri1* and double mutants between *cpd*/*bri1* and flowering-time mutants, *ga1* and *ld*. Under long days, the *cpd-10* and the *bri1-201* flowered slightly late with a very similar flowering time (Fig. 3.12). However, the analyses of double mutants impaired in the gibberellin pathway (*ga1*) and the autonomous pathway (*ld*) revealed differences between the *cpd* and *bri1* mutants. The *bri1* mutant has a much stronger effect on the flowering of the *ga1* and *ld* mutants than that of the *cpd* mutant (Fig. 3.12, 3.13). At the same time that the *cpd* mutation may affect the flowering of the *ld* mutant, through the same molecular mechanism – enhancement of *FLC* expression – as does the *bri1* mutant (Fig. 3.14). Additionally, the *bri1* mutant was less responsive to a vernalization treatment than was the *cpd* mutant (Fig. 3.15). All together, it can be concluded that

some effects of *bri1* are shared with *cpd*, therefore they are likely to be specific to physiological function of BRs. For instance, the severe reduction in the levels of endogenous BRs delays flowering of the autonomous mutant *ld* and increases *FLC* expression, which also seems to be the main cause of the late flowering of the *bri1 ld* mutant. At the same time, the influence on flowering time of *bri1* is greater than this of the *cpd* mutant, even though the *bri1-201* mutant, used in this experiment, exhibits a less severe dwarfism (which is one of the phenotypes associated with BR-deficiency) compared to the *cpd-10* mutant. It appears, that the BR-deficiency is only partially the basis of the delay in flowering of the *ld* mutant by *bri1*, and additional *BRI1*-specific factors should be considered. Furthermore, some effects, for example weaker response to vernalization or enhancement of *ga1* mutant, seem to be only specific to *bri1*, indicating that its function in the floral transition is exerted through both BR-dependent and BR-independent processes. The *cpd* mutant does not significantly enhance the *ga1* mutant, which could suggest that *cpd* and *ga1* function in the same pathway. The late-flowering phenotype of the *ga1 bri1* double mutant, the *bri1* mutant appears to be additive to *ga1*, indicating independence of the GA- and *BRI1*-pathways. Also, the fact that vernalized *bri1* flowers later than vernalized *cpd* provides further evidence for differences in mechanisms underlying the late flowering of these two mutants. Since the exposure to cold efficiently accelerates flowering of the *bri1 ld* mutant, *bri1* does not appear to be affected in the vernalization response itself. It should also be noticed that in these experiments weak allele of the *bri1* mutation (*bri1-201*) was used, resulting in a flowering time phenotype comparable to that of the *cpd* mutant. It can therefore be expected that if the stronger *bri1-202* allele was used, it would flower later than *cpd*. This seems to be true, because under greenhouse conditions, *bri1-202* (with a more severe dwarfism) flowered later than *bri1-201* (data not shown). Collectively, this supports the broader role for *BRI1* apart from a BR-related function.

One explanation for the different effects of *bri1* and *cpd*, is the possibility that BRI1 does not exclusively function as a BR-receptor, *i.e.* that it binds and transduces signals from other ligands. This is particularly interesting, because the tomato *BRI1* has been shown to have a dual function: as a receptor of BRs and as one for systemin, a peptide hormone that mediates systemic wound responses (Sheer and Ryan, 1999,

2002; Sheer *et al.,* 2003; Montoya *et al.,* 2002). Systemin is only present in the Solaneae subtribe of the Solanaceae family, and therefore the possibility that the Arabidopsis *BRI1* binds this particular peptide can be excluded (Constable *et al.,* 1998). However, other LRR-receptor kinases have also been shown to bind peptides (for example FLS2 binds the flg22 peptide) (Gomez-Gomez *et al*., 2000; 2001; Bauer *et al*., 2001; Wang and He, 2004). Perhaps, Arabidopsis BRI1 not only binds BRs, but also an unknown developmentally or environmentally regulated peptide or secreted protein. This peptide could serve as a "hormone" in the floral transition. It is also possible that BRI1 functions as a broad receptor for multiple steroids. In such a scenario, bioactive BRs would be absent in the *cpd* mutant, but other sterols such as less active precursors of BL, or non-BR steroids, such as sitosterol, stigmasterol *etc.,* would be present and bind to BRI1 to regulate some specific physiological processes (Clouse, 2000). However, in the light of the very severe dwarf phenotype of the *cpd* mutant this seems unlikely, unless the effects of these ligands on the controlled processes are opposite to those of BL.

It is possible that BRI1 could function as a co-receptor in a non-BL-binding receptor complex. Since receptor-like kinases (RLKs), including BRI1, are known to function as homo- or heterodimers, another RLK might bind an unknown ligand, but requires BRI1 to transduce its membrane-transmitted signal (Karlova et al., 2006; Albrecht et al, 2005; Johnson and Ingram; 2005, Wang et al., 2005 a, b). Again, such an interaction between a hypothetical RLK and BRI1 could be regulated developmentally by controling the presence of the ligand and the expression of the co-receptor in a stage- and/or tissue-specific manner. Information provided by environmental cues, such as light quality or intensity, temperature, *etc.* could be another way to control the activity of an alternative BRI1-containing receptor complex.

A third way to describe the observed discrepancies in flowering behaviors of the BR-deficient mutant and the BR-receptor mutant is to postulate the existence of a BR-binding receptor, different than BRI1. This implies that the effects of the loss of the *CPD* and *BRI1* activity on flowering time would be independent from each other. It has been reported that opposite to the BR-deficient mutant, *bri1* mutants accumulate bioactive BRs such as brassinolide, castasterone and typhasterol to high

levels (Noguchi *et al.,* 1999). This is caused by the lack of repression of expression of BR-biosynthesis genes: *CPD* and *DWF4,* which normally occurs upon BL-binding and signal transduction (Vert *et al.,* 2005). Hence, if other BR-receptor exists, in addition to BRI1, such a hypothetical receptor would be exposed to an excess of brassinosteroids, in the absence of the functional BRI1, resulting in a stronger signal flow in the pathway downstream of this receptor. Theoretically, this function could be fulfilled by two BRI1-like receptors, named *BRL1*, and *BRL3*. Both have been shown to bind BL and function as a BR-receptor. The expression of *BRL1* and *BRL3* is restricted to non-overlapping subsets of vascular tissue, where they regulate differentiation and growth (Caño-Delgado *et al.,* 2004, Zhou et al., 2004). Thus, the putative *BRI1*-independent effects would be restricted to vascular tissue. Since the *det2* (BR-deficient) and *bri1* mutants show similar alteration in the vascular tissue, it seems that the role of *BRL1* and *BRL3* can be excluded (Caño-Delgado *et al.,* 2004). Nevertheless, it is still possible that an unknown, perhaps cytoplasmic BR-receptor exist. Such receptors, functioning as nuclear transcription factors, are well described in animals, where they mediate most steroid responses (*eg*. Aranda and Pascual, 2001). Increased activity of such receptor could manifest itself in two manners: global change in the chromatin structure or specific alteration of expression of a subset of BR-regulated genes. If the first situation would be true, the increase of *FLC* expression in the double *bri1 ld/fca/FRI* would be a "side effect" of a general disturbance in the control of chromatin state, rather than a specific upregulation of *FLC* expression.

The analyses of flowering time and *FLC* expression in the steroid mutants, and the double mutants between these steroid-deficient mutants and the *ld/fca/FRI* lines compared to the *cpd ld/fca/FRI* and *bri1 ld/fca/FRI* should help to clarify whether other steroids also regulate the autonomous and *FRI* pathways. Alternatively, direct testing BRI1 for binding of non-BR steroids would resolve whether BRI1 can function as a broad steroid receptor.

The construction of the triple *cpd bri1 ld* mutant, in turn, would provide a genetic background which simultaneously has reduced endogenous levels of BRs and non-functional BRI1 and mutation in the autonomous pathway. Such mutant could prove to be useful in resolving the hypothesis of the additional BR-receptor.

To identify potential receptor kinases that interact with BRI1, the yeast-two-hybrid screen with the BRI1-kinase domain used as a bait. This approach was successfully applied to isolate BAK1, which functions as a BRI1-coreceptor (Nam and Li, 2002). Alternatively, the putative BRI1-containing complexes could be isolated from the transgenic plants expressing the tagged BRI1 protein and immunoprecipitating with a antibodies raised against the used tag. Proteins that would coprecipitate with BRI could be later identified by means of MALDI-TOF/MS technology. Such approach has been utilized to isolate members of the SERK1 complex (Karlova *et al.,* 2006).

Furthermore, examining the global chromatin state, the level of methylation and various histone modifications in the wild type plants and *bri1, ld*, and *bri1 ld* mutants would answer whether *bri1* affects specifically the expression of *FLC,* or if it causes a general disturbance in chromatin structure.

In summary, the results described here provide evidence that both the BR-receptor BRI1 and brassinosteroids themselves are important factors of floral-regulating network in Arabidopsis. Surprisingly, however, *BRI1* exerts its effects on flowering only partially through the BR-regulated pathway. The nature of *BRI1*-specific effects on flowering time remains to be investigated.

3.3. Genetic analyses of hormone interactions in the floral timing

The role of many well-known phytohormones in the control of flowering time in Arabidopsis has not been extensively studied. The function of gibberllins (GAs) in the floral transition is the best documented example. GAs are believed to promote flowering in Arabidopsis, and their function is particularly important in the absence of the inductive photoperiodic signal, *i.e.* under short days (Wilson *et al.,* 1992; Blazquez *et al.,* 1998). Abscisic acid (ABA) has recently been demonstrated to delay flowering, at least partially, through interaction with the autonomous pathway (Razem *et al.,* 2006; Achard *et al.,* 2006). A potential role for brassinosteroids (BRs) was suggested based on the modest late-flowering phenotype of BR-deficient mutants, but to date it has not been investigated in detail (Chory *et al*., 1991; Azpiroz *et al.,* 1998).

It has been reported that for many physiological processes, hormone-signaling pathways do not function as separate entities. They interact at various levels within the signalling process to ensure an appropriate biological response (Gazzarrini and McCourt, 2003). A well-described example of such hormone interactions is the regulation of seed germination, in which GAs and BRs have been shown to function antagonistically to ABA to break dormancy and promote germination (Koornneef and Karssen, 1994; Steber and McCourt, 2001). It was thus hypothesized that these three hormones would genetically interact in the regulation of the floral transition. Here, the potential interactions between the BR-, the GA- and ABA- regulated pathways in the control of the transition from vegetative to reproductive development were studied. The impact of genetic lesions in the BR, GA, and ABA biosynthetic pathways was directly tested to assess their interactive network. Double mutant combinations defective in the biosynthesis of ABA, BRs, and GA were constructed and their flowering time was measured. Also, plants that overexpress genes encoding rate-limiting enzymes in biosynthesis of ABA, BRs or GAs were generated in order to increase the endogenous levels of the respective hormones, and their flowering time was investigated. Finally, the expression of key flowering-time genes was analyzed in plants with altered endogenous levels of BRs, GAs, and ABA.

3.3.1. Flowering-time analyses of double mutant combinations deficient in BR, ABA and GA

3.3.1.1. Construction of double mutant combinations deficient in BR, ABA and GA

To construct double mutants deficient in BR, ABA, and GA, in all possible combinations, the following single mutants were selected: *constitutive photomorphogenesis and dwarfism* (*cpd*)*, gibberellin deficient1* (*ga1*)*,* and *abscisic acid deficient2* (*aba2*). The chosen *cpd, ga1,* and *aba2* mutants are blocked in the biosynthesis of BRs, GAs, or ABA, respectively (Fig. 3.16), and each have been shown to contain extremely low levels of the respective hormone (Szekeres *et al*., 1996; Zeevaart and Talon, 1992; Leon-Kloosterziel *et al*., 1996, Cheng et al., 2002). The double mutants (*cpd ga1, aba2 ga1, aba2 cpd*) were generated by crossing the respective single mutants. The double homozygous lines were obtained in the F2 progeny by genotyping or by phenotypic identification, as described in Chapter 2.

3.3.1.2. Analyses of genetic interactions between the *ga1, cpd,* and *aba2* mutants

The obtained double mutants (*aba2-2 ga1-3, ga1-3 cpd-10, aba2-2 cpd-10*) together with single *ga1-3, aba2-2, cpd-10* mutants and Ws wild-type were subjected to flowering-time analyses under long days (Fig. 3.17). Since the observed phenotypes were modest, the Student's t-test was used to test the significance of observed differences between genotypes (Table 3.2). Under long days in the greenhouse, the *aba2* mutant was early flowering and the *ga1* and *cpd* mutants were slightly delayed compared to WS (Fig 3.18). The double *aba2 ga1* mutant exhibited earlier flowering compared to single *ga1*, and later than the single *aba2* mutant. The double *aba2 cpd* mutant was almost as late flowering as the single *cpd* mutant, but at the same time it had similar flowering time to the wild type WS plants. The flowering time of this double mutant was not significantly different from the single *cpd* nor wild type, suggesting that its flowering time is somewhere between wild type and *cpd.* In contrast, the double *cpd ga1* mutant flowered slightly later than the single *cpd* mutant

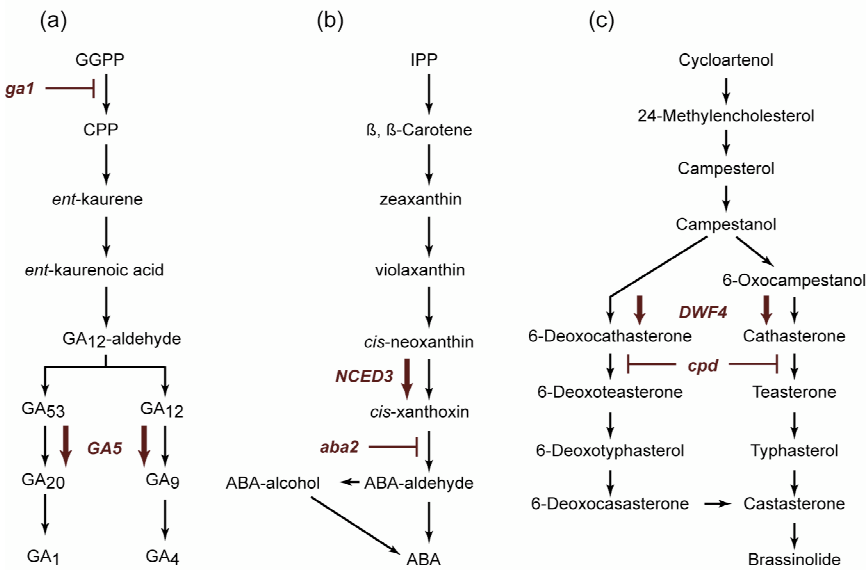

Figure 3.16. Simplified biosynthetic pathways in Arabidopsis for gibberellins (a), ABA (b), and brassinolide (c). The biosynthesis mutants used in this study and sites of their lesions are shown. Also, the biosynthetic genes overexpressed to increase the levels of respective hormones are indicated. The *ga1* mutant is impaired in the first stage of GA-biosynthesis: the cyclization of geranylgeranyl diphosphate (GGPP) to copalyl diphosphate (CPP). The *aba2* mutant is blocked at the cis-xanthoxin to ABA-aldehyde conversion. The conversion of 6-Deoxocathasterone/Cathasterone to 6-Deoxoteasterone/teasterone does not occur in the *cpd* mutant. The *GA5* gene encodes a GA 20-oxidase that catalyzes the formation of the GA_{20} and GA_9, the final precursors of the bioactive GAs. The *NCED3* encodes 9-*cis*-epoxycarotenoid dioxygenase that catalyzes the oxidative cleavage of a 9-*cis* isomer of epoxycarotenoid (9-*cis*-violaxanthin or 9'-*cis*-neoxanthin) to form xanthoxin. The *DWF4* gene encodes a 22- hydroxylase (CYP90B1) that catalyzes the conversion of 6-Oxocampestanol/Campestanol to 6-Deoxocathasterone/Cathasterone. IPP, Isopentenyl pyrophosphate. ABA, abscisic acid. Adapted from "Plant Growth and Development. Hormones and Environent", *Ed.* LM Srivastava (2001).

Figure 3.17. Phenotypes of the wild-type WS, the single *cpd-10, ga1-3, aba2-2* mutants and the *ga1-3 cpd-10*, *aba2-2 cpd-10* and *aba2-2 ga1-3* double mutants. Plants were grown under long days (16h light/8h darkness) in the greenhouse. Pictures were taken when wild-type plants were flowering (the bolt was 1 cm high). The white bar indicates 1 cm.

Table 3.2. Student's t-test for the flowering time of the hormone single and double mutants.

Genotype 1	Genotype 2	P value
WS	*aba2-2*	0.003123 *
WS	*cpd-10*	0.026681 *
WS	*ga1-3*	0.000035 ***
WS	*aba2-2 cpd-10*	0.638560 ø
WS	*aba2-2 ga1-3*	0.012466 *
WS	*cpd-10 ga1-3*	0.000483 **
aba2-2	*aba2-2 cpd-10*	0.000231 *
cpd-10	*aba2-2 cpd-10*	0.068565 ø
aba2-2	*aba2-2 ga1-3*	0.000025 ***
ga1-3	*aba2-2 ga1-3*	0.009909 *
cpd-10	*cpd-10 ga1-3*	0.000359 **
ga1-3	*cpd-10 ga1-3*	0.063013 ø

Listed here are pairs of genotypes, which were compared. P values for each pair are provided.
No significant difference P>0.05 ø statistically significant differnces: P<0.0001***, P<0.001**, P<0.05*

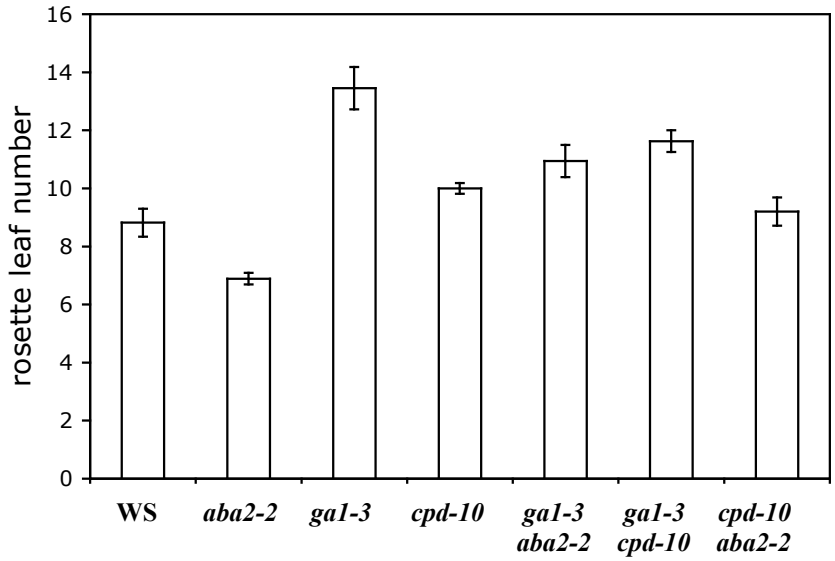

Figure 3.18. Flowering-time analyses of the wild-type WS, the single *cpd-10, ga1-3, aba2-2* mutants and the *ga1-3 cpd-10*, *cpd-10 aba2-2,* and *ga1-3 aba2-2* double mutants. Plants were grown under long days (16h light/8h darkness) in the greenhouse. Flowering time was measured as rosette leaf number at bolting. Between 5 and 17 plants were scored. Error bars represent SE. Two experiments were performed, and a representative result is shown. Note that the *aba2* mutant flowered earlier, and the *ga1* and *cpd* single mutants were delayed compared to the wild type. The flowering time of the double *cpd aba2* mutant was in the range between the flowering of the single *cpd* mutant, and wild type. The *ga1 cpd* mutant flowered later than the single *cpd* mutant, and its flowering time was not significantly different compared to that of the single *ga1* mutant. The *ga1 aba2* double mutant flowered later than the single *aba2*, but earlier than the *ga1-3* mutant. The significance of the differences in flowering times among mutants was analyzed using Student's t-test. The results of the analyses are presented in Table 3.2.

and was not different from the single *ga1* under the greenhouse condition.

3.3.1.3. Gene expression analyses in the double mutant combinations deficient in BRs, ABA and GA

In order to gain more insight into the molecular mechanisms of altered flowering in the BR-/ABA- and GA- deficient mutant, and the double mutant combinations, the expression of selected flowering-time genes was examined. Plants were grown aseptically on agar plates under long-day conditions, and samples were taken after 10 days. Gene expression was monitored using RT-PCR, the primers specific to EF1-α were used as a control (Fig. 3.19). The *aba2* mutant clearly had higher levels of *FT* and *CO* transcripts and slightly increased expression of *SOC1*. The *ga1* mutant was not found to have pronounced alterations in the expression of the tested genes, except for a subtle decrease in *CO* expression. The *cpd* mutant was found to have increased levels of *FLC* mRNA, and decreased expression of *FT,* and slightly lower levels of *SOC1*. Interestingly, the double *aba2 cpd* mutant had markedly reduced expression of *FLC* and *SOC1.* The levels of *FT* did not appear to be affected, and the expression of *CO* was marginally increased. Surprisingly, the *ga1 aba2* mutant had elevated levels of *FLC* mRNA, and reduced expression of *SOC1* and to some degree of *FT* and *CO*. In the *ga1 cpd* double mutant, elevated expression of *FLC*, and reduced levels of *FT* and *SOC1* transcripts were observed. Thus, the single and double mutants tested here exhibited an altered expression of key flowering-time genes, but different genes were affected in each mutant. This implies that the balance between these hormones is necessary for the appropriate timing of the floral transition.

3.3.2. Flowering-time analyses of plants with elevated expression of rate-limiting enzymes in the biosynthesis of ABA, BRs and GAs

In order to further examine the role of BRs, GAs and ABA in the floral transition, plants with elevated expression of key genes in the biosynthesis of

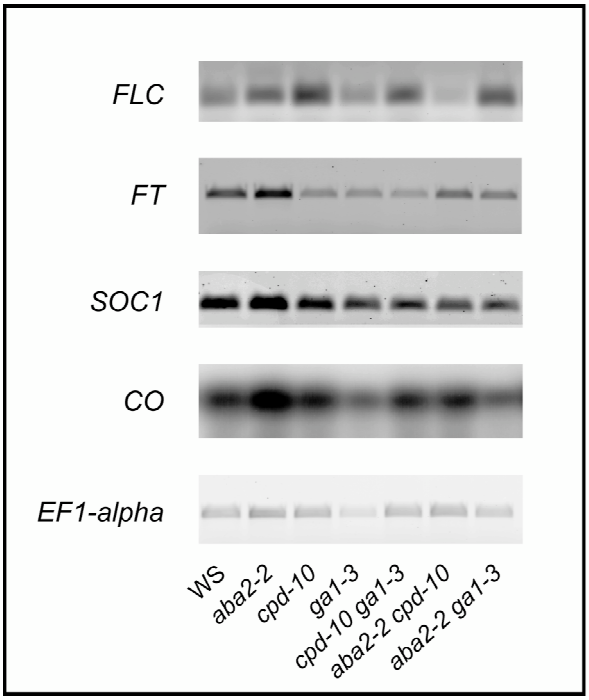

Figure 3.19. Expression levels of *FLC, FT, SOC1* and *CO* in the single and double hormone mutants as monitored by RT-PCR. Primers specific for the elongation factor 1-alpha gene were used as a control. Note that the *aba2* mutant had increased levels of *FT*, *CO*, and *SOC1*. The *cpd* mutant contained high levels of *FLC* transcript and decreased amounts of *FT* and *SOC1* mRNAs. The *ga1* mutant did not exhibit any striking molecular phenotypes; *CO* expression is slightly reduced in this mutant. The double *aba2 cpd* mutant was found to have decreased amounts of *FLC, SOC1* and *FT* and slightly increased level of *CO*. The *aba2 ga1* double mutant had higher levels of *FLC*, and reduced expression of *SOC1, FT* and *CO*. In the *ga1 cpd* double mutant elevated expression of *FLC* was observed, and reduction in the amount of *FT* and *SOC1* transcripts.

each respective hormone were constructed and their flowering time, as well as the expression of key flowering time genes were studied.

3.3.2.1. Construction of *35S::DWF4*, *35S::GA5* and *35S::NCED3* lines

To study the effect of overexpression of rate-limiting enzymes in the BR, GA, and ABA biosynthesis on the timing floral transition, the *DWF4,* the *GA5,* and the *NCED3* genes were chosen, respectively. (Fig. 3.16). The selected genes have been previously shown to cause an increase in the endogenous level of the respective

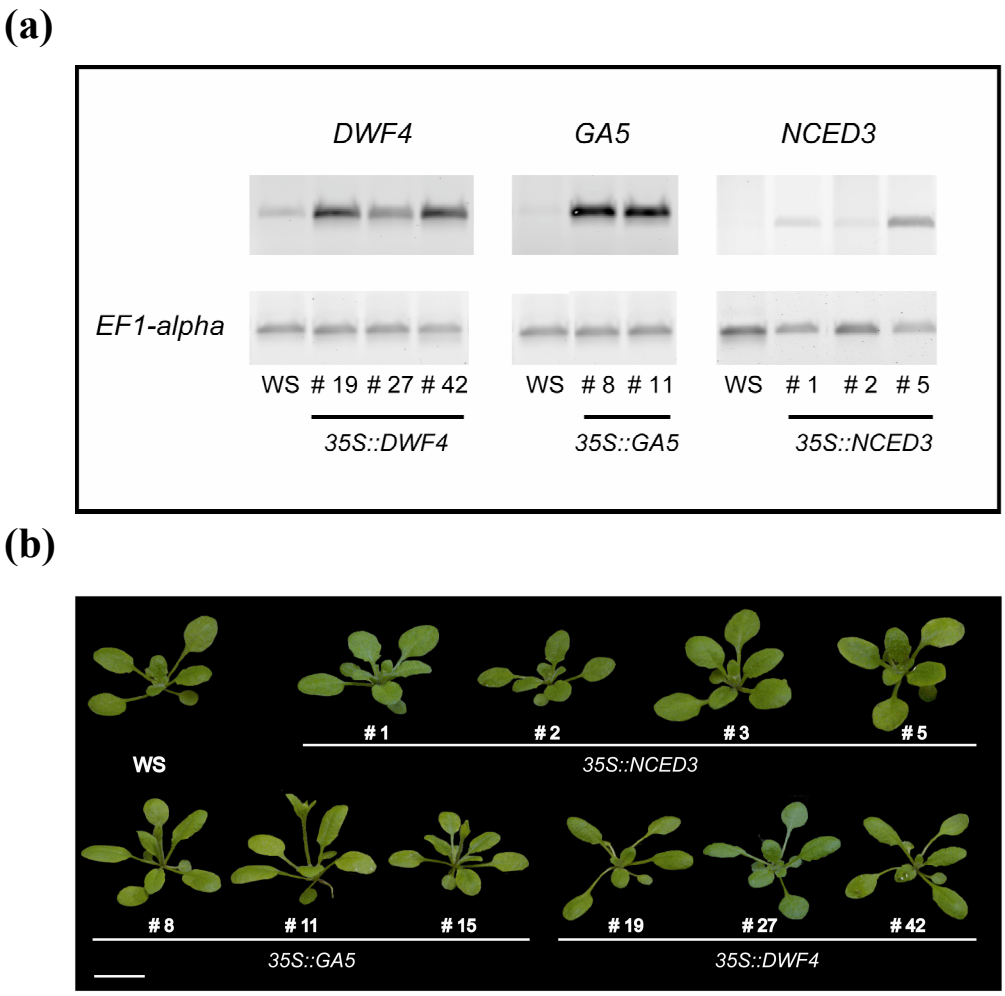

Figure 3.20. Transgenic lines harbouring *35S::DWF4*, *35S::GA5* and *35S::NCED3* constructs. (a) Overexpression was confirmed by RT-PCR with primers specific for *DWF4*, *GA5* and *NCED3*. Primers specific for the elongation factor 1-alpha gene were used as a control. Representative lines are shown. All lines tested showed overexpression of the gene of interest. (b) Phenotypes of 3-weeks-old plants grown under long days (16 h light/8 h darkness) in the greenhouse. The white bar indicates 1 cm.

hormone or its precursors when overexpressed (Choe *et al.,* 2001; Huang *et al.,* 1998; Coles *et al.,* 1999; Iuchi *et al.,* 2001). These hormone biosynthetic genes were misexpressed under control of the Cauliflower Mosaic Virus 35S promoter, which enabled expression of the genes to high levels. The construction of the *35S::DWF4*, *35S::GA5* and *35S::NCED3* vectors and the obtained transgenic lines harboring the constructs is described in detail in Chapter 2. The overexpression of the genes of interest was confirmed using RT-PCR with gene-specific primers. As a control, RT-

PCR with EF1-α-specific primers was performed (Fig 3.20 a). The obtained transgenic lines were also analyzed for expected phenotypes that are attributed to the overproduction of the respective hormone (Choe *et al.,* 2001; Huang *et al.,* 1998; Coles *et al.,* 1999; Iuchi *et al.,* 2001, Wang *et al.,* 2001). As expected, the *35S::GA5* plants had longer hypocotyls, lighter-green leaves, and increased stem elongation. Also as expected, the *35S::DWF4* lines showed increased growth, longer hypocotyls, greater height, elongated rosette leaves and petioles resembling the *35S::BRI1* plants. The ABA-overproduction features found in *35S::NCED3* plants included increased seed dormancy and elevated expression of tested ABA- and drought-inducible genes (data not shown). Visual phenotypes of the 3-week-old transgenic lines are shown in Fig. 3.20 b. In conclusion, the constructed *35S::DWF4, 35S::GA5* and *35S::NCED3* lines displayed phenotypes specific for the overproduction of the respective hormones. These were thus found to be suitable for flowering-time studies.

3.3.2.2. Flowering time analyses of *35S::DWF4*, *35S::GA5* and *35S::NCED3* lines

The obtained *35S::DWF4*, *35S::GA5* and *35S::NCED3* lines were subjected to flowering-time analyses under long and short days (Fig. 3.21). The flowering time of similar *35S::GA5* genotypes has already been reported (Huang *et al.,* 1998; Coles *et al*., 1999), and the results described here are therefore confirmatory. The differences in flowering times among mutants were analyzed using Student's t-test. As expected, three representative lines of the *35S::GA5* flowered early under both long and short days, confirming the GA-overproduction phenotype ($P<0.0001$). Neither *35S::DWF4* nor *35S::NCED3* exhibited a consistently altered flowering time. Under long days, only one out of three *35S::DWF4* lines flowered significantly early (line # 42, $P<0.05$). Under short days, none of the lines showed reproducible changes in flowering. Perhaps, this was due to an adult-stage suppression of the overexpression phenotype that was observed in lines # 19 and # 42. The *35S::DWF4* line #27, which expresses the *DWF4* gene to lower levels (Fig. 3.3.4 a) compared to lines # 19 and # 42, did not exhibit suppression and flowered slightly later under short days (around 5 leaves difference), but only in one experiment was this difference significant. The

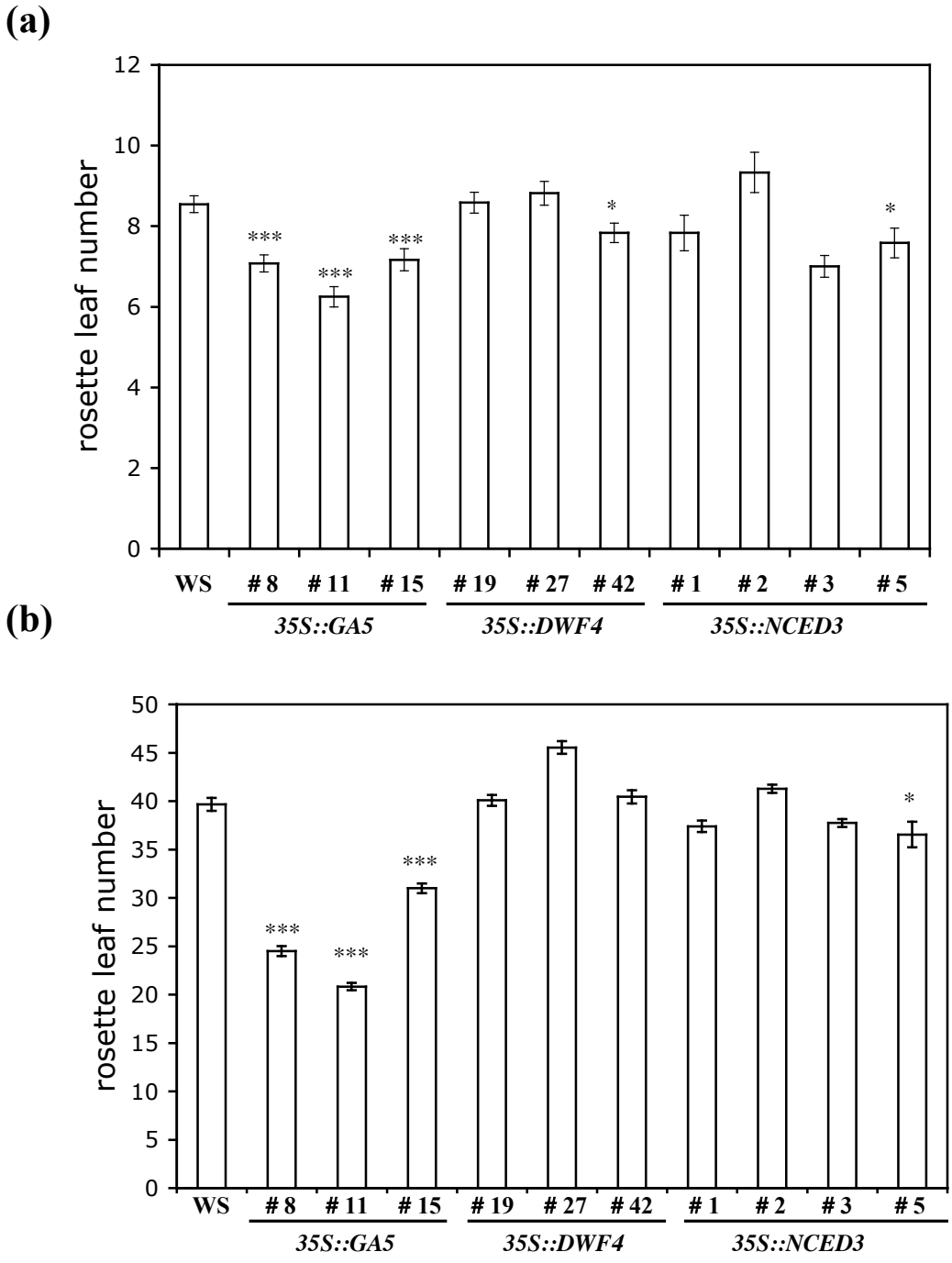

Figure 3.21. Flowering time of the transgenic lines that overexpress GA-, BR- and ABA-biosynthetic genes: *GA5, DWF4* and *NCED3*, respectively. Two experiments were performed, and the result from one experiment is shown. (a) Long-day conditions (as described in Fig. 3.3.2). All three *35S::GA5* lines flowered significantly earlier than wild type plants. Only one *35S::DWF4* line (out of three lines tested) flowered significantly slightly earlier in a reproducible manner. Also one *35S:NCED3* line (#5) showed early flowering that was significant and reproducible. (b) Short days (8h light/16 h darkness). All three *35S::GA5* lines flowered significantly earlier. None of the *35S:DWF4* line exhibited reproducible, significant alteration in flowering time. Perhaps due to an observed adult-stage suppression of the *35S:DWF4*-overexpression phenotype (as noted for visual growth traits) for lines # 19 and 42. One *35S:NCED3* line (#5) flowered significantly early. Student's t-test was applied to test for the differences in flowering time, P<0.0001***, P<0.05*.

35S::NCED3 line #5 was the only out of four *35S::NCED3* lines that showed accelerated flowering in a reproducible and significant manner ($P<0.05$) under both conditions. Interestingly, line #2, which expressed *NCED3* to the lower levels than other tested *35::NCED3* lines tested flowered later than wild type, but this difference was only significant in one out of two experiments performed. Hence, only GAs seem to have a limiting role for the flowering-time control, while ABA and BRs do not appear to be limiting.

3.3.3. Discussion

The understanding of the role of phytohormones in the control of the floral transition in Arabidopsis has predominantly focused on gibberellins as known promoters of flowering, and in particular, under a non-inductive photoperiod (Wilson *et al.,* 1992; Blazquez *et al.,* 1998). The function of other hormones is only recently being uncovered, *eg.* ABA has been shown to inhibit flowering through interaction with the autonomous pathway (Razem *et al.,* 2006; Achard *et al.,* 2006). The promotive role of BRs in the transition to reproductive development has been suggested based on the modest late-flowering phenotype of BR-deficient mutants (Chory *et al*., 1991; Azpiroz *et al.,* 1998). No detailed studies were to date have reported on BR-regulated flowering.

It is believed that signaling pathways do not function as entirely separate modules, and there is cross talk between pathways at various levels of their signal transduction. This also seems to be true for the hormone-signaling pathways that interact in diverse ways to bring about coordinated physiological responses (Gazzarrini and McCourt, 2003). Here, the potential genetic interaction among BR-, GA- and ABA- regulated pathways in the control of the floral transition were studied. Double mutant combinations deficient in BRs, GAs, and ABA were constructed and analyzed for flowering time and expression of key flowering-time genes. These studies were supplemented with an examination of plants that exhibited a BR-, GA- and ABA- overproduction phenotype due to the overexpression of genes encoding the rate-limiting biosynthetic enzymes *DWF4*, *GA5* and *NCED3*, respectively.

The analyses of the flowering phenotypes of double *aba2/ga1/cpd* mutant combinations did not reveal any strong genetic interactions (Fig. 3.18). Based on the

flowering behavior of the double *aba2 ga1* mutant compared to the respective single mutants, it can be concluded that these two genes function mostly independently. No significant difference in flowering time between the double *cpd aba2* and single *cpd* mutants was observed, which suggests that the BR-deficient mutant is epistatic to the ABA-biosynthesis mutant. However, since the double *cpd aba2* also did not differ from wild type, it indicates that *aba2* acts partly independently from the *cpd* mutation. Finally, the lack of statistical difference between the *cpd ga1* double mutant and the *ga1* single mutant implies that these BR- and GA-deficient mutants are in the same genetic pathway. Taken together, the relationships between the studied hormonal pathways in the control of flowering time are complex and cannot be put into a simple linear pathway. Perhaps, a part of this complexity is caused by reciprocal, differential regulation of the hormone biosynthetic genes by various hormone-signaling pathways, as it has been shown that in seedlings BR and GA antagonistically regulate the accumulation of mRNAs of the GA-regulated *GASA1* and *GA5* genes (Bouqin *et al*., 2001).

Based on the expression-analyses of hormone mutants, it can be concluded which flowering pathways are modulated by BRs and ABA (Fig. 3.19). The fact that the *cpd* mutant exhibited increased expression of *FLC*, and reduced *FT* and *SOC1* expression, is suggestive that BRs modulate the autonomous pathway. This result is consistent with findings described in 3.1 and 3.2 [where it was described that both a mutation in the BR-receptor (*bri1*) and *cpd* enhance the late-flowering phenotype of the autonomous mutant *ld,* by enhancing the expression of *FLC*]. The *aba2* mutant was found to have higher levels of *CO, FT,* and perhaps *SOC1*, which could suggest that ABA modulates the photoperiod pathway. Similarly to the flowering-time analyses, it is difficult to make straightforward conclusions based on the results of the gene expression studies in the double hormone mutants. For example, the *aba2 cpd* mutant exhibited lower expression of *FLC* compared to the *cpd* single mutant, and this was even lower than the wild type. Yet this double mutant flowered almost as late as the BR-deficient plant. Surprisingly, at the same time *aba2 cpd* had low levels of floral-pathway integrators *FT* and *SOC1* (which are targets of repression by *FLC*). This could suggest that other unknown factors are involved in the repression of these genes in *aba2 cpd*. It might be that the *cpd* mutation establishes an extended juvenile

phase, during which a plant lacks the competence to respond to floral promoting signals. In summary, the single and double hormone mutant combinations tested in this study exhibited an altered expression of key flowering-time genes, indicating that the balance between these hormones is necessary for an appropriate timing of the floral transition.

The analyzed transgenic lines that overexpressed the *NCED3* and *DWF4* genes did not exhibit strong flowering phenotypes (Fig. 3.21). In general, *35S::NCED3* plants were slightly earlier flowering than wild type, except one line that tended to be delayed in flowering. Even though that the effects were mild and statistically insignificant (as tested with Student's t-test), the observed general trend differs from what has been published recently regarding the role of ABA in the floral transition. It has been reported that exogenous ABA application decreases the growth rate and delays flowering (Achard *et al.,* 2006; Razem *et al*., 2006) through upregulation of *FLC*. As mentioned earlier, the *35S::NCED3* plants exhibited increased expression of *NCED3* and an ABA-overexpression phenotype including delayed germination and growth, and activation of some ABA-regulated genes. At the same it has been shown that overexpression of this ABA-biosynthetic gene results in an elevation of the endogenous levels of ABA (Iuchi *et al*., 2001). Thus, the observed phenotypes in the generated *35S::NCED3* plants are expected to result from an increased ABA content. However, to further confirm that these plants overproduce ABA, ABA levels could be directly measured in these plants. One way to explain the discrepancy between the observed phenotypes is that overexpression of NCED3 not only increases the levels of ABA, but also of other metabolites that affect flowering. NCED3 catalyzes the oxidative cleavage of a 9-*cis* isomer of epoxycarotenoid (9-*cis*-violaxanthin or 9'-*cis*-neoxanthin) to form xanthoxin. Perhaps the excess of the enzyme leads to an unspecific catalytic activity that results in the appearance of alternative reaction products. Alternatively, not all xanthoxin might be converted to ABA, and its excess could promote flowering through an unknown mechanism. It should also be considered that ABA is a "stress hormone", because ABA levels increase upon stress treatment and it mediates the response to drought and other stresses (Finkelstein *et al*., 2002). At the same it has been reported that drought accelerates flowering (Levy and Dean, 1998). Hence, it might be that at low

concentrations ABA inhibits flowering, and after reaching a certain threshold, it induces the floral transition. This could explain the mildly early flowering observed in some of the *35S::NCED3* lines. Further detailed studies on the mechanism of drought- and ABA-induced flowering are required to resolve this issue. Similarly, no pronounced flowering phenotype was detected when *35S::DWF4* lines were analyzed (Fig 3.21). Under long days, only one line was found to flower statistically earlier, therefore overproduction of BRs seems not to affect flowering under this condition. Under short days, two tested lines exhibited an adult-stage suppression of the overexpression phenotype, and consequently it was impossible to draw any conclusions from this experiment. The third *35S::DWF4* line maintained a weak overexpression and flowered later than wild type, but only in one experiment this result was significant. Thus, it is possible that BRs repress flowering under a non-inductive photoperiod, but the data presented here are insufficient to make such conclusion. Anyhow, the observed phenotypes were modest, indicating that BRs do not have a limiting or major role in floral promotion. The *35S::GA5* plants clearly flowered earlier under both photoperiodic conditions, confirming the importance of GAs in the control of the transition to flowering (Fig. 3.21).

To clarify the relationships between the examined hormones in regulation of the transition to reproductive growth, it would be worthwhile to measure the flowering behavior of the hormone-deficient mutants harbouring the *35S::DWF4*, *35S::NCED3* and *35S::GA5* constructs. For example, the *35S::DWF4 ga1-3* and *35S::GA5 cpd* would allow to define the epistatic relation between the BR- and GA-regulated pathways in the control of the floral transition.

The mild phenotype of the BR-/ABA-deficient mutant and the lack of a significant flowering phenotype in the transgenic lines that overexpress the *DWF4* and *NCED3* genes, leads to a conclusion that these hormonal pathways are necessary for proper timing of the floral transition, but are themselves insufficient to trigger or inhibit the transition. GA in turn, seems to be a “master” hormone over ABA/BR. The overexpression of the *GA5* gene results in a plant with a clear early-flowering phenotype, regardless of the photoperiod, confirming the promotive role of this hormone. Finally, the major role of gibberellins and the supporting function of ABA and BRs can also be inferred from the analyses of the double hormonal mutants.

3.4. Analysis of the integration of the BR pathway on the LFY promoter

Genetic pathways that regulate the transition to flowering ultimately activate a small subset of genes, the so-called floral pathway integrators. They integrators activate expression of floral meristem-identity genes, which trigger the floral transition. *LEAFY* (*LFY*) is particular in its flowering-time control: it functions not only as a floral meristem identity gene, but also as a floral pathway integrator (Blazquez *et al.,* 1997). This has been demonstrated by Blazquez and Weigel, who showed that both the photoperiod and the gibberellin pathway activate the *LFY* promoter, but this happens through separable *cis* elements (Blazquez and Weigel, 2000). Thus, the *LFY* promoter seems to be a good system to test the convergence of various floral promotive pathways, and it could be used to identify additional factors controlling the transition to reproductive growth, for instance through the chemical-genetics approach.

Identification of the brassinosteroids/BR receptor signaling pathway as a component of the flowering-regulating network raised the question on which downstream targets this pathway is integrated. In particular, the *LFY* promoter seemed to be one of the possible sites for BR pathway convergence. To test whether BRs activates the *LFY* promoter, the *LFY::LUCIFERASE* reporter system has been constructed and introduced in to the transgenic line that overexpress the BR-biosynthetic genes, *DWF4*. The usefulness of the obtained reporter construct has been validated and finally the effect of *35S::DWF4* transgene on the *LFY* promoter activity has been investigated.

3.4.1. Construction of the *LFY::LUC+* reporter

To monitor the transcriptional activation of the *LFY* promoter, the modified firefly luciferase gene (*LUC+*) has been chosen as a reporter gene of its activity. The advantages of using the luciferase gene are the short half-life of the protein, an even shorter half-life of the activity of the enzyme, and possibility to image living plants over their life, as the assay is non-destructive. The important feature of using luciferase is that its activity can be quite precisely quantified (Millar *et al.,* 1992). All

these features make the luciferase an excellent system for non-invasive *in vivo* monitoring of the rate of the transcription activation of the promoter of choice.

The *LFY* promoter was defined as 3.7 kb region upstream of the ATG site for the *LFY* gene till the last bp before the STOP codon of the neighbouring gene. This fragment fully overlaps with the *LFY* promoter used by Blazquez *et al*. (1997). The same authors, however, reported that the *LFY* promoter they used, seemed to not entirely reproduce the *LFY* RNA pattern, probably due to the lack of some regulatory cis elements (Blazquez *et al.,* 1997). Therefore, to include the potentially missing motifs, the promoter used in this study contained additional 1.4 kb. The construction of the *LFY::LUC+* transgenes was described in detail in Chapter 2.

3.4.2. Analysis of the *LFY::LUC+* expression pattern during the vegetative phase

The pattern of *LFY* expression, as well as of the activation of the *LFY* promoter by different floral promoting signals, has been previously determined in detail by Blazquez and colleagues (Blazquez *et al*., 1997), and therefore the activity of constructed *LFY::LUC+* transgene was compared to results described by these authors.

The *LFY::LUC+*-harboring plants were grown on agar, under long- and short-day conditions, and the promoter activity was measured throughout the vegetative growth phase. The spatial and temporal activity of the *LFY* promoter was assessed by *in vivo* monitoring luminescence emission by means of the single-photon-counting liquid nitrogen-cooled CCD camera. Since the *LFY* RNA pattern during vegetative growth was only described in detail in plants grown under short days, this condition was chosen to thoroughly test the expression pattern of the constructed *LFY::LUC+* transgene. Three independent transgenic lines were tested and representative results are shown. In general, in plants grown under short days, the highest luminescence intensity was detected in the central part of the plants, including young leaves and basal parts of the older leaves (Fig. 3.22). This expression pattern was observed during most of the vegetative phase, although in the early stages of growth, considerable luciferase was also detected in cotyledons and leaves. Under long days, a similar

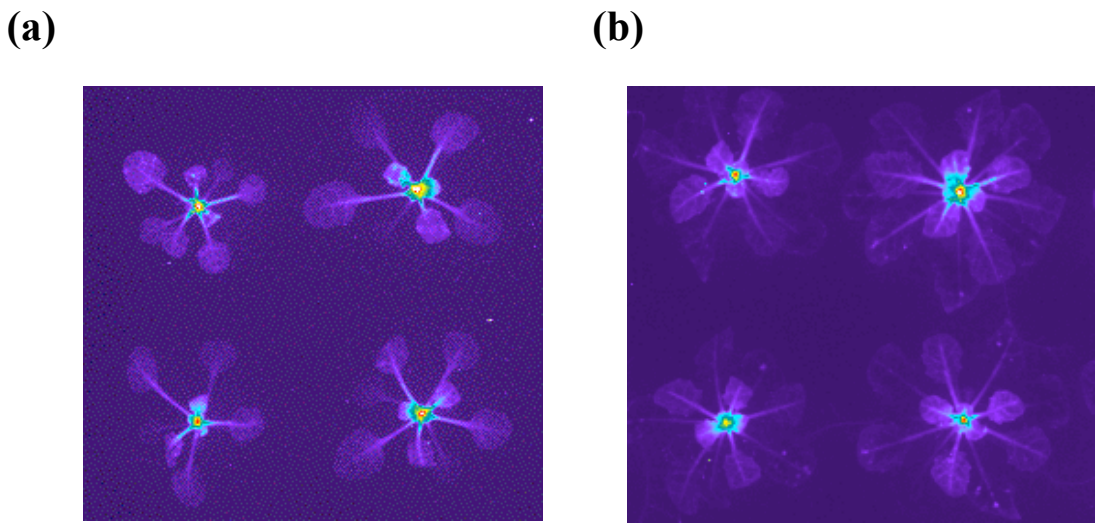

Figure 3.22. *LFY::LUC+* expression pattern in transgenic plants grown for 18 days (a) or for 40 days (b) under short days. The luminescence images of plants are shown. Plants were grown on MS-agar plates and imaged from the top using CCD camera. The image is processed in a false-color scale: blue denotes the lowest signal, while white and red indicate the highest intensity of luminescence. Note that the strongest luminescence intensity is found in the central apical region of the plants.

expression was observed, though restriction of the expression to the central part of the plant was less apparent. The observed pattern of the *LFY* promoter activity mostly resembles that of the endogenous *LFY* mRNA detected by in situ hybridization (Blazquez *et al*., 1997).

3.4.3. Time-course of *LFY::LUC+* expression

To further examine the usefulness of the *LFY::LUC+* construct, the developmental time-course of promoter activity under non-inductive and inductive photoperiods was examined to test whether it mirrors the results obtained in the *GUS*-reporter studies. Under long days, the *LFY::LUC+* activity increased rapidly, while in short day-grown plants, the level of *LFY::LUC+* activity remained low (Fig. 3.23 a) and increased only slightly with time. Moreover, when plants were grown under short days for 21 days and then transferred to long days, a rapid and clear increase in the luminescence intensity was detected (Fig. 3.23 b). Thus, it seems that *LFY::LUC+* is strongly upregulated by long days, which is in agreement with the observations

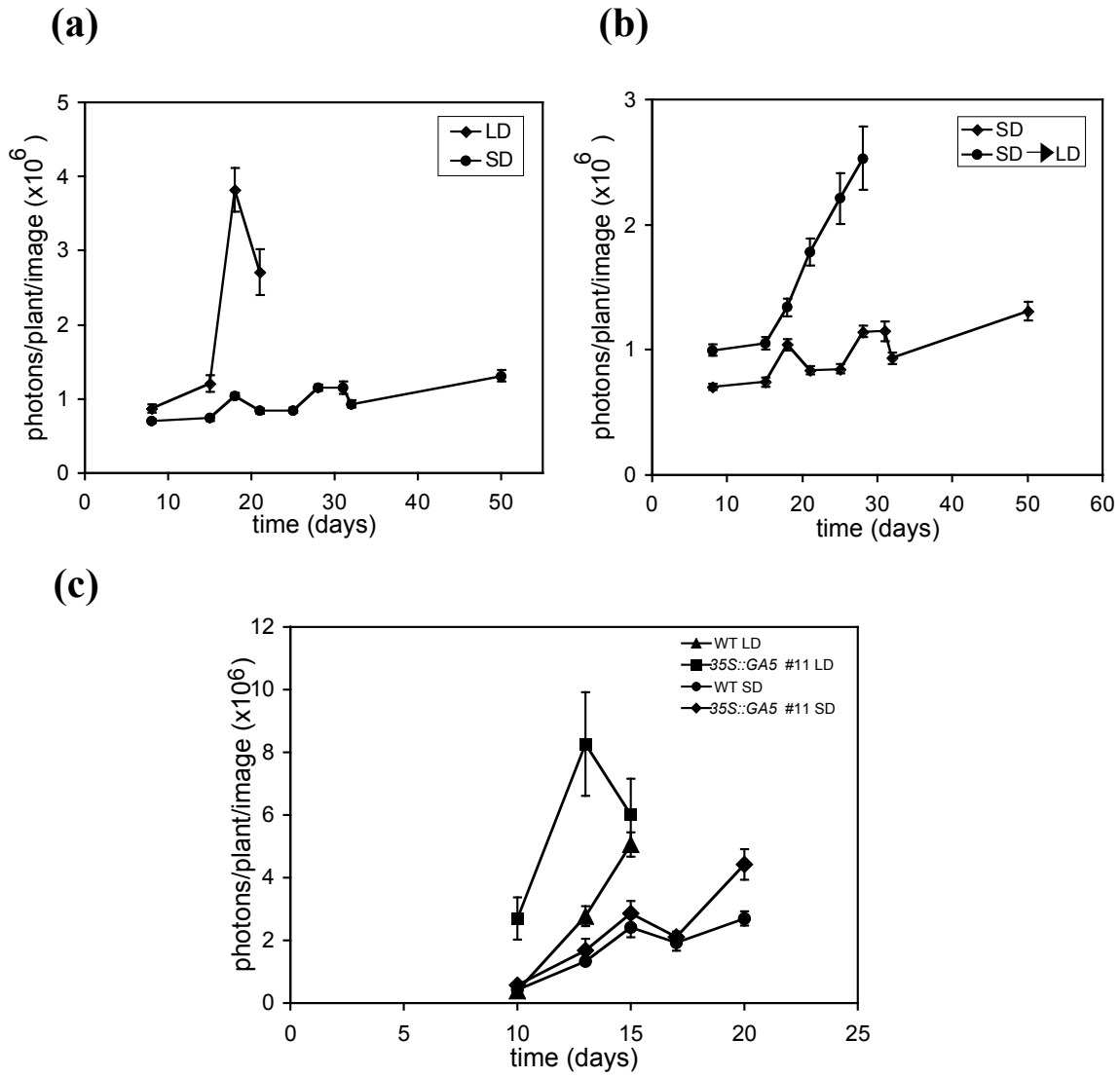

Figure 3.23. Time-course of *LFY::LUC+* expression. *LFY::LUC+* activity was monitored using CCD camera, which registered total luminescence. Luminescence of single plants was quantified using MetaMorph imaging software. Between 3-12 plants per genotype were scored. Error bars indicate SE. Representative results are shown. (a) Comparing *LFY::LUC+* expression under long and short days. Under long days *LFY::LUC+* increased rapidly after 10 days, while in the short-days grown plants it remained low. (b) The *LFY::LUC+* reporter is activated by long days. Plants were grown under short days or under short days and after 21 days transferred to the inductive photoperiod. Upon transfer to long days quick increase in the luminescence intensity was detected. (c) The *LFY::LUC+* activity is elevated in the *35S::GA5* plants compared to the wild type, both under long- and short-days condition.

reported by Blazquez and colleagues (Blazquez et al., 1997). However, they claimed that under short-day conditions, the *LFY* promoter activity increased gradually till it reached a certain threshold level that is sufficient for the transition to flowering, and in case of the *LFY::LUC+*, only a subtle increase was observed (Fig. 3.23 c). This

raised the question whether the *LFY::LUC+* transgene can respond properly to the floral promotive signal. It has been shown that under non-inductive photoperiods flowering is promoted by gibberellin, the application of this phytohormone accelerates flowering and it correlates with enhanced activation of the *LFY* promoter (Blazquez *et al.,* 1998). Therefore, the effect of gibberellin on the activity of *LFY::LUC+* was examined. One representative reporter line was crossed to plants that overexpressed a GA-biosynthethic enzyme, *GA5* (*35S::GA5* transgenic plants). The overexpression of *GA5* leads to GA-overproduction phenotype, which mimics exogenous application of GA, including early flowering under both short and long days (GA-overproducing lines are described in the Chapter 3.3). The 35S::*GA5* lines was found to have higher *LFY::LUC+* activity than wild-type plants under both non- and inductive photoperiods, indicating that the tested reporter construct responds correctly to gibberellins (Fig. 3.23 c).

3.4.4. The steady-state levels of *LFY* mRNA are increased in the *35S::GA5* plants under long days

To test whether the activity of the *LFY::LUC+* reporter reflects the levels of the *LFY* transcript, semi-quantitative RTPCR was performed The *LFY* mRNA abundance was measured in 10-days old seedlings of wild-type plants , and *35S::GA5* transgenic lines grown under long- and short- day conditions. Under long days *35S::GA5* and showed higher levels of *LFY* expression compared to wild-type plants. The extremely low level of *LFY* mRNA in the short-day grown plants did not allow reliable quantification (Fig. 3.24). Nevertheless, the results for long-day grown plants indicate that the activity of the *LFY::LUC+* transgene mirrors the levels of the endogenous *LFY* transcript.

3.4.5. Time-course of *LFY:LUC+* expression in the *35S::DWF4* background

The obtained *LFY:LUC+* reporter was clearly activated by the photoperiod and gibberellin pathways, in a similar manner as reported by Blazquez *et al.,* (1997)

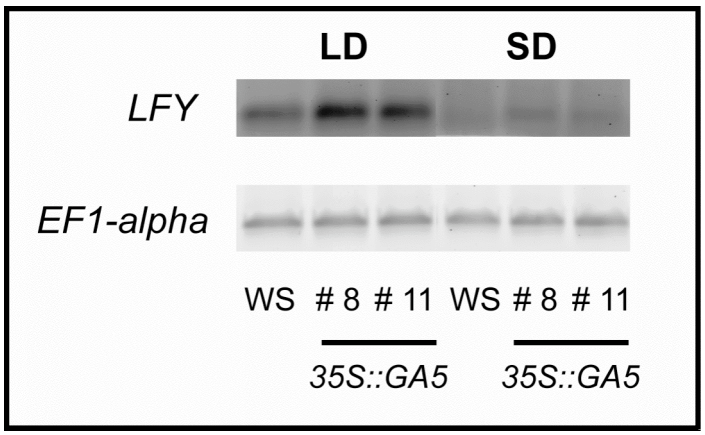

Figure 3.24. Expression level of *LFY* in 10days-old seedling of the *35S::GA5* transgenic lines compared to wild type WS, as monitored by RT-PCR. Primers specific for the elongation factor 1-alpha gene were used as a control. LD denotes long days, SD denotes short days. Note that in the *35S::GA5* plants grown under long days increased expression of *LFY* was observed. Similar trend was observed for plants grown under non-inductive photoperiod, however, due to extremely low levels of *LFY* mRNA it was impossible to make strong conclusion

indicating that it might be a suitable system for testing other potential floral regulating signals.

To test whether brassinosteroids can upregulate the *LFY* promoter, the reporter *LFY:LUC+* line was crossed to plants that overexpress a BR-biosynthetic gene, *DWF4* resulting in a BR-overproduction phenotype (*35S::DWF4* transgenic plants are described in Chapter 3.3). The obtained BR-overproducing plants harboring *LFY:LUC+* were used to analyse the time-course of the *LFY* promoter activity under both long- and short-day conditions. Under long days, the results were quite variable: in the same *35S::DWF4* lines, detected luminescence was found to be either higher or lower in the independent experiments (Fig. 3.25 a, b). Surprisingly, *LFY:LUC+* appeared to be repressed in all *35S::DWF4* lines tested when plants were grown under non-inductive photoperiods (Fig. 3.25 c, d).

3.4.6 Discussion

Diverse floral promotive pathways ultimately converge on a small number of genes, termed floral pathways integrators (Boss *et al.,* 2004). One of these genes,

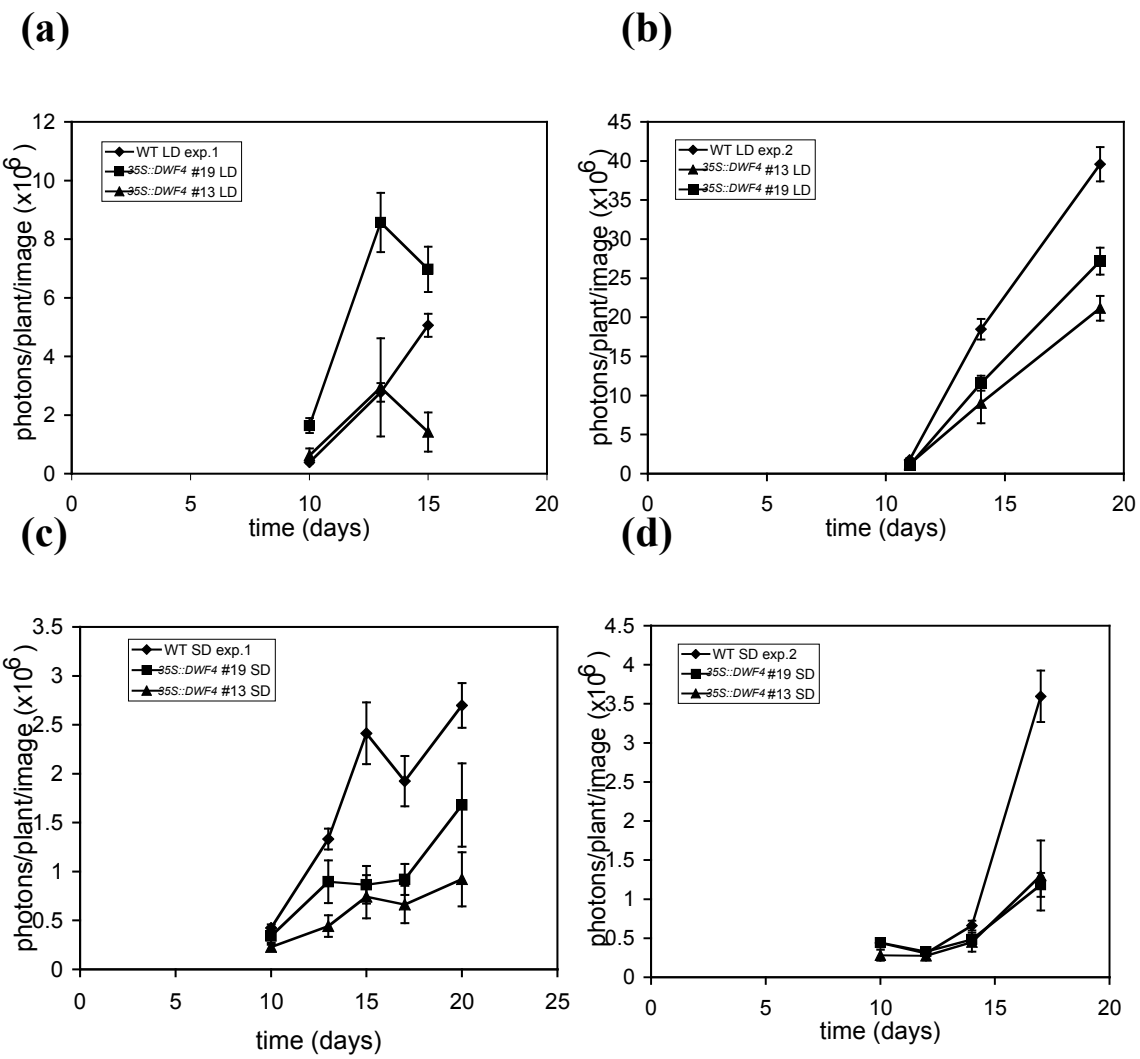

Figure 3.25. Time-course of *LFY::LUC+* expression in the *35S::DWF4* lines. Results from two independent experiments are shown. *LFY::LUC+* activity was monitored using CCD camera, which registered total luminescence. Luminescence of single plants was quantified using MetaMorph imaging software. Between 3-12 plants per genotype were scored. Error bars indicate SE. (a,b) *LFY::LUC+* expression under long days in two independent *35S::DWF4* lines, obtained in two independent experiment. Note that results vary between the shown experiments. (c,d) The *LFY::LUC+* expression under short days in two independent *35S::DWF4* lines, obtained in two independent experiment. The *LFY* promoter activity is lower in the *35S::DWF4* lines under the non-inductive photoperiod.

LFY, has been shown to integrate signals of the photoperiod and the gibberellin pathways, and likely also for the autonomous pathway (Blazquez and Weigel, 2000; Aukerman *et al*., 1999). This integration happens on the level of the promoter through distinct cis element, therefore the *LFY* promoter appeared to be good system to study the convergence of various flowering pathways, and to identify additional factors that regulate the transition to the reproductive phase. Here, the *LFY:LUC+* reporter

system was constructed, validated, and the effects of the elevated expression of the BR-biosynthetic gene, *DWF4* on the *LFY* promoter activity was assessed.

The expression pattern of the *LFY::LUC+* transgene was examined throughout development in plants grown under inductive and non-inductive photoperiods using the CCD camera. This imaging system did not allow to define the expression pattern with the precision comparable to *in situ* hybridization. Nevertheless it was possible to describe the global expression pattern of the *LFY::LUC+* transgene. During the vegetative phase, the *LFY::LUC+* expression was detected in the in the shoot apical region of the plant, including the basal parts of the leaves. The activity of the *LFY::LUC* was also detected in newly emerging leaves, but the luminescence disappeared from older leaves (Fig. 3.22). In the early stages of the development, luminescence could be detected in cotyledons and leaves. This effect was more pronounced under long days. In general, the observed *LFY::LUC+* expression pattern resembles the *LFY::GUS* and *LFY* RNA described by Blazquez and colleagues (Blazquez *et al.,* 1997). Since these authors did not describe in detail how the *LFY* expression pattern changes throughout the development under long days, it is difficult to conclude whether the observed activity of the *LFY* promoter in the cotyledons and early leaves is biologically relevant or an artifact caused by a reporter system used. Also, it has not been reported by any detection method whether LFY expression in cotyledons was tested. It should be noticed, however, that the low levels of *LFY* RNA could be already detected in the 3-day-old long-day grown seedlings (Bradley *et al.,* 1997). The biological relevance of *LFY* expression in such young seedlings is unclear. Hence it might be that the *LFY* promoter is marginally activated early in the development, and it can be detected with the *LFY::LUC+* reporter due to the strong enhancement of the signal.

The next step to validate the *LFY::LUC+* reporter system was to monitor the time-course of resulting luminescence. Strong induction under long days, and rapid increase in the expression upon transfer to inductive photoperiod (Fig. 3.23 a, b), indicates that the promoter responds to the long-day pathway in a similar manner as it was reported for the *LFY::GUS* (Blazquez *et al*., 1997). Under short days, in turn, only a very low signal of the *LFY::LUC+* was detected (Fig. 3.23 b). This is partially in agreement as to what was reported by Blazquez and colleagues, but in contrast to

these authors, no gradual increase in the promoter activity was detected under this condition. This variation may reflect different properties of GUS and luciferase reporters: GUS protein is extremely stable, while the half-life of the luciferase enzymatic activity is very short making it more suitable reporter gene for transcriptional activation studies (Hondred *et al.,* 1999; Millar *et al.,* 1992). It seems probable that the detected slow increase in the GUS activity under short days (Blazquez *et al.* 1997) is partially due to the accumulation of GUS protein. Nevertheless, the higher signal of *LFY::LUC+* activity in the *GA5*-overexpressing plants (which are early flowering and exhibit GA-overproducing phenotype) compared to the wild-type plants, suggests that *LFY* promoter is activated by gibberellins and this can be detected using the constructed reporter transgene (Fig. 3.23 c). Also, the levels of *LFY* transcript were increased in the *35S::GA5* transgenic plants grown under long days compared to wild type, further supporting that the *LFY::LUC+* activity can reflect the levels of endogenous *LFY* (Fig. 3.24). Direct detection of *LFY* levels by RT-PCR in the 10-days-old plants grown under short days were below the detection level. Therefore, it is unclear what was the level, of *LFY* expression in the *35S::GA5* plants grown under non-inductive photoperiod. Interestingly, when levels of *LFY* transcript were analyzed in shoot apices of 4-6 week-old wild-type plants grown under short days, no apparent increase in *LFY* expression was observed, and *LFY* mRNA was still barely detectable (data not shown). This suggests that under short days, *LFY* does not gradually accumulate to the threshold levels that can trigger transition, but it is expressed in similar low levels throughout the vegetative phase and only increases upon the floral transition when floral primordial are formed. If this is true, then the results obtained with *LFY::GUS* by Blazquez and colleagues are in question. However, more sensitive detection method of gene expression is required to test this hypothesis.

The *LFY::LUC+* transgene was tested for responsiveness to brassinosteroids by introducing it to plants harboring the *35S::DWF4* construct. The *35S::DWF4* plants exhibited the BR-overproduction phenotype, but their flowering was not significantly altered. It is difficult to make a conclusion whether/how the *LFY* promoter activity was regulated in these lines grown under long days, there was a high level of variation within the same lines between experiments performed (Fig.

3.25 a, b). This may imply that under the inductive photoperiod, the *LFY* promoter is not a direct target of BR-pathway, which is in agreement with the flowering-behavior of the *35S::DWF4* lines. Under short days, the *LFY::LUC+* activity was consistently reduced (Fig. 3.25 c, d). However, no significant differences in flowering time were observed under this conditions. Hence, BR might repress *LFY* expression, but the results presented here do not allow one to make a firm conclusion. The analyses of *LFY* mRNA levels and the *LFY::LUC+* expression in the BR-deficient mutants and exogenous application of brassinosteroids should help to clarify whether/how this class of phytohormones regulates expression of *LFY*.

4. General conclusion and perspectives

The timing of the transition to flowering is regulated by multiple endogenous and environmental factors that together interact in bringing about this appropriate physiological response. Genetic analyses of late-flowering mutants resulted in defining at least four genetic pathways that promote flowering: the photoperiod, the autonomous, the vernalization, and the gibberellin pathways (Boss *et al.*, 2004; Putterill *et al.,* 2004). It appears that despite the quite intensive studies, additional factors regulating the floral transition still await characterization. This might be particularly true in the case of genes that have a modulating activity and cannot be easily identified in direct genetic screens. In order to isolate genes, whose function in a floral transition is less pronounced, suppressor or enhancer screens in the sensitized genetic background could prove to be useful. As a result of such genetic screen, two independent alleles of *bri1* were isolated as strong enhancers of the late-flowering phenotype of the autonomous mutant *luminidependens* (*ld*). *BRI1* encodes an LRR-RLK that functions as a receptor for BRs, thus the result of the screen indicated that *BRI1* or BRs could play a role in the floral timing. The aim of this thesis was to define and compare the roles of *BRI1* and BRs in the floral transition. The studies were extended with the examination of potential genetic interactions between BRs, GAs, and ABA in the control of the transition to the reproductive phase in Arabidopsis.

4.1. Defining the *BRI1* pathway in the control of flowering time

Genetic analyses of diverse double mutant and gene-expression studies presented and discussed in chapter 3.1, led to conclusion that *BRI1* establishes a previously unknown genetic pathway that regulates the timing of floral transition. The *BRI1* pathway appears to function mostly independently from the gibberellin, the photoperiod, and the vernalization pathways. At the same time, *BRI1* genetically interacts with the autonomous pathway to repress the expression of the strong repressor *FLC*. Given that the *bri1* single mutant has only a modest late-flowering phenotype, while the autonomous mutants or *FRI* plants have much more pronounced phenotypes, *BRI1* probably has an assisting role to the autonomous pathway in

repressing *FLC*. This also implies that the *BRI1*-pathway does not function to directly promote flowering, but by enabling repression of a strong floral repressor, which introduces the competence in the SAM to respond to floral inductive signals, such as photoperiod.

4.2. Comparing the role of *BRI1* and BRs in floral transition

The clear flowering phenotype of the BR-receptor mutant *bri1* raised a question whether its phenotype is specific to BR-deficiency or rather a result of the pleiotropic nature of the mutation. This was investigated by comparing the phenotypes of the BR-deficient mutant *cpd* with the *bri1* mutant. Based on the results from these studies (described and discussed in chapter 3.2), it was proposed that *BRI1* likely exerts its function on flowering through both BR- dependent and independent activity. Postulated models to describe *BRI1*-specific and BR-independent effects on flowering, and experiments to test some of the proposed hypotheses were also discussed (in chapter 3.2).

4.3. Genetic analyses of hormone interactions in the floral timing

The role of BRs in the control of flowering time was also studied in the context of its putative interactions with the GA- and ABA-regulated pathways (chapter 3.3). Examination of double mutant combinations deficient in BRs, GAs and ABA did not reveal strong genetic interactions amongst these hormones. Based on the increased expression of *CO, FT,* and *SOC1* in the *aba2* mutant, it was proposed that ABA modulates the photoperiod pathway. The increased levels of *FLC,* and reduced *FT* and *SOC1* levels, further support that the BR pathway interacts with the autonomous pathway. Furthermore, a differentially altered expression of key flowering-time genes (*CO, FT, SOC1, FLC*) in single and double hormone mutant combinations tested in this study, points to the importance of the balance between these hormones for the appropriate timing of the floral transition. The modest flowering phenotypes of transgenic lines that overexpress *35S::DWF4*, and *35S::NCED3,* leading to BR- and ABA-overproduction phenotype, respectively,

together with the mild flowering phenotypes of BR-/ABA-deficient mutants indicates that these two hormones do not have a major promotive role in the control of the transition to flowering. GA in turn has clearly a limiting function in the transition to flowering, as reported previously and confirmed in these studies (Wilson *et al.,* 1992; Blazquez *et al.,* 1998).

4.4. Analysis of the integration of the BR pathway on the LFY promoter

Identification of the brassinosteroids/BR receptor signaling pathway as a component of the flowering-regulating network raised the question on which downstream targets this pathway is integrated. In particular, the *LFY* promoter seemed to be one of the possible sites for BR pathway convergence, since it has been shown that both the photoperiod and the gibberellin pathways regulate this promoter through separate cis elements (Blazquez and Weigel, 2000). Attempts were made to verify whether the BR-pathway converges on the *LFY* promoter. (Chapter 3.4) For this purpose, the *LFY::LUC+* reporter system was constructed, validated, and tested for BR-induction. No clear activation of the *LFY* promoter in the BR-overproducing lines was observed, suggesting that the LFY promoter is not activated by the BR pathway. However, further studies are needed to make a final conclusion whether BRs regulate *LFY* expression.

4.5. Future perspectives

4.5.1. Unravelling the molecular mechanism of *BRI1*/BRs *FLC* regulation

The main finding of the studies reported here is that *BRI1* regulates flowering time, mostly through regulation of the expression of *FLC*. Presented results (chapter 3.1), however, are insufficient to conclude whether the *BRI1*-pathway actively represses *FLC* or functions to maintain the repression established by other factors. The observed decrease of *FLC* expression throughout development in the autonomous mutant *ld,* suggests that specific, perhaps age-related mechanisms, might be involved to repress *FLC* thus enabling flowering. Since *bri1* strongly enhances *ld*, the *BRI1*-

pathway could provide a signal for the induction of *FLC* repression. This hypothesis could be tested by overexpressing *BRI1* in genetic backgrounds that contain elevated levels of *FLC*, such as autonomous mutants or *FRI*-carrying lines. If *BRI1* directly induces *FLC* expression, such plants would flower earlier. Similarly, the BR-deficient mutant *cpd* was shown to have increased levels of *FLC* and an enhanced expression of this repressor in the *ld* mutant, though the flowering behavior of the double *cpd ld* was less severe that of *bri1 ld*. This suggests that the regulation of *FLC* is a common feature for the BR- and *BRI1*- pathways. Hence, a parallel hypothesis that BRs can repress *FLC* expression could be formulated and tested by exogenous application of BRs or increasing endogenous levels of BRs (by overexpressing rate-limiting BR-biosynthetic enzymes) in the high-*FLC*-containing lines mentioned above. The proposed experiments would also help to characterize the relationship between the BR- and BRI1-regulated flowering.

A substantial body of literature has appeared in recent years regarding the role of *FLC* chromatin modification in flowering-time control (reviewed by He and Amasino, 2005; Sung and Amasino, 2005). Many factors that function to activate or repress *FLC* expression do so by introducing a range of modifications, mostly to histones, but also at the DNA itself. In future experiments, it would be interesting to define the molecular/biochemical mechanism of *FLC* regulation by BRs/*BRI1*. The first step to address this problem could be to trace known changes in the *FLC* chromatin structure (specific modification of specific histones are being introduced in a defined order) throughout development of single *bri1, cpd, ld*, and double *cpd ld,* and *bri1 ld* mutants, compared to wild-type plants. In such a way, it could be assessed which steps in *FLC* regulation are missing in the respective mutants, *i.e.* it would help to resolve whether *FLC* repression is properly established, if this repression is maintained, *etc*. Again, comparing *cpd* and *bri1* mutants should help to clarify a basis of differences between these two mutants.

Another approach to study the epigenetic regulation of *FLC* by BRs/*BRI1* is to test directly whether any of the known nuclear components of the BR-signaling pathway binds to the *FLC* chromatin (*eg.* BES1, BZR1, BIM1) using chromatin immunoprecipitation (ChIP) and tagged versions of these proteins. Also, by looking for differences in *FLC* chromatin between mutants, one could find candidate

processes or molecules that function in complexes introducing modifications of interest, and test them for interaction with the BR-signaling proteins.

In this study, only the effects of two elements of BR-signaling on flowering time were tested: i) a loss of function of the main receptor and ii) BR-deficiency. It would be interesting to see what the effect is (compared to *cpd* and *bri1*) of a reduction in activity of components of BR-signaling downstream of *BRI1* on the flowering time of autonomous mutants. For example, as mentioned above, *BZR1, BES1*, and *BIM1* are excellent candidates for testing the most downstream elements of BR-induced processes. Since *BES1* and *BZR1* function partially redundantly to each other and to four other members of this gene family, loss-of-function mutations in these genes do not show visual phenotypes (Wang *et al.,* 2002, Yin *et al.,* 2002). A similar situation is observed with the *BIM* family (Yin *et al.,* 2005). Thus, to observe a potential flowering phenotype, the effect of the loss-of-function of the whole gene family could be investigated with regard to *FLC*-expression responses and subsequent physiological alterations in flowering. This could be achieved by obtaining a multiple mutant or by introducing an RNAi targeted construct against the gene family of interest into plants. The latter option is faster, and provides the possibility to study a dosage effect in the reduction of function. This experiment would help to assess the effect of a loss of BR-induced, BRI1-mediated signaling on flowering and *FLC* expression.

4.5.2. Putative link between BRs and aging

One of the features of BR-deficient mutants is a delayed onset of senescence (Chory *et al*., 1991, Vert *et al.,* 2005). The same has been observed for *bri1* mutants (Vert *et al.,* 2005). Hence, one could imagine that the transmission of an unknown age-related signal(s) is blocked in these mutants. Perhaps, one of the functions of such signal is not only to initiate senescence at an appropriate developmental time, but also to initiate downregulation of *FLC* (if this has not happened yet), to ensure that the plant can enter the reproductive phase, followed by further developmental stages. If this is true, then the effect of *cpd/bri1* on flowering would not be exclusive to BRs, but it would rather be a less specific delayed aging effect. Perhaps a similar enhancing

activity on flowering time of autonomous mutants could be mimicked by combining them with mutants in developmental aging, such as *ore4* (Woo *et al.,* 2002). To verify whether the onset of senescence is impaired in *bri1/cpd ld* compared to *cpd*, *bri1, ld* and wild-type plants, the induction of aging-specific senescence-associated genes, such as *SAG12* (Gan and Amasino, 1997) could be tested. Interestingly, *cpd* still flowers late even if *FLC* levels are decreased, as occurs in the double *cpd aba2* mutant. This suggests that a high level of *FLC* is not a main cause of late flowering of this mutant, but a "side effect" induced by this mutation. This might be due to a lack the competence to respond to floral promotive signals. This seems to be in an agreement with the hypothesis that impairment in age-regulated processes causes the flowering phenotype of the *cpd* mutant. Further analyses are required to make final conclusions.

4.5.3. BRs/BRI1 and light signaling in the control of floral transition

Many brassinosteroid-deficient mutants have been initially identified in genetic screens for deetiolated/constitutive morphogenic phenotypes when grown in darkness. In addition to their morphological phenotypes, they also exhibited expression of light-induced genes in darkness, indicating that BRs are implicated in light signaling. A comparable phenotype was observed for dark-grown *bri1*. Moreover, it has been reported that the response to exogenously applied brassinosteroids differs depending on the light quality and quantity (Nemhauser and Chory, 2002). Also, the analysis of *bas1-D*, a gain-of-function mutant, which has reduced levels of BRs due to increased inactivation of BRs, revealed that *bas1-D* can fully suppress *phyB* in red light, only partially suppress *phyA* grown in far-red light, and partially suppress *cry1*. This provided a link between levels of BRs and light signals (Neff *et al.,* 1999). Furthermore BRs have been involved in repressing the phyA-mediated very low fluence response (VLFR) (Luccioni *et al.,* 2002). All these reports point to a role of BRs in light-mediated processes. As described in the introduction, various features of light, such as light quality or photoperiod, are important factors regulating the timing of floral transition (Boss *et al.,* 2004; Searle and Coupland, 2004). It would be interesting to test whether BRs/BRI1 modulate

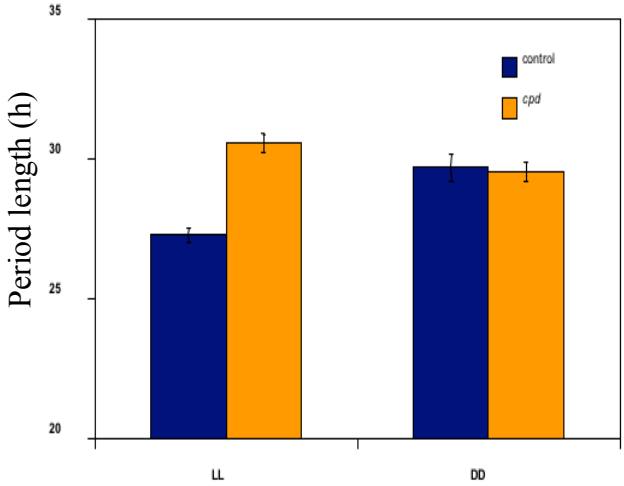

Figure 4.1. BR-deficient mutant has altered circadian rhythms. Shown is period length of *CCR2::LUC* rhythms in the *cpd* mutant compared to the wild type control measured under constant light (LL) or under constant darkness (DD). *cpd* lengthened periodicity under LL conditions. From Hanano *et al.,* submitted.

these pathways. It appears, based on the more-than-additive phenotype of *bri1 gi,* compared to *gi,* that *BRI1* indeed interacts to some extent with the photoperiod pathway. The phenotype of the *cpd gi* double mutant awaits characterization. The photoperiod pathway promotes flowering in response to the inductive day-length. The photoperiodic response integrates environmental signals (light) to the time-keeping mechanism (circadian clock) to regulate the floral transition (Searle and Coupland, 2004). Thus interaction with the photoperiod pathway could occur on many levels throughout the signal transduction pathway. The BR-deficient mutant *det2* has been previously shown to have an altered circadian rhythm, suggesting that BRs can modulate the circadian system. Experiments performed in the Davis group confirmed that BRs play a role in maintaining the periodicity and robust rhythms of the clock system (Hanano *et al.,* submitted, Fig. 4.1). Preliminary findings were that *bri1* has a comparable circadian phenotype to *cpd*, as assayed via circadian-regulated *CCR2::LUC+* reporter construct (Fig. 4.2). Thus, a component of the late-flowering of BR-deficient and *bri1* mutants might be altered circadian processes. The relationship between altered circadian rhythms in the BR-deficient and *bri1* mutants, and the effects of these mutations on *FLC* expression awaits further investigation.

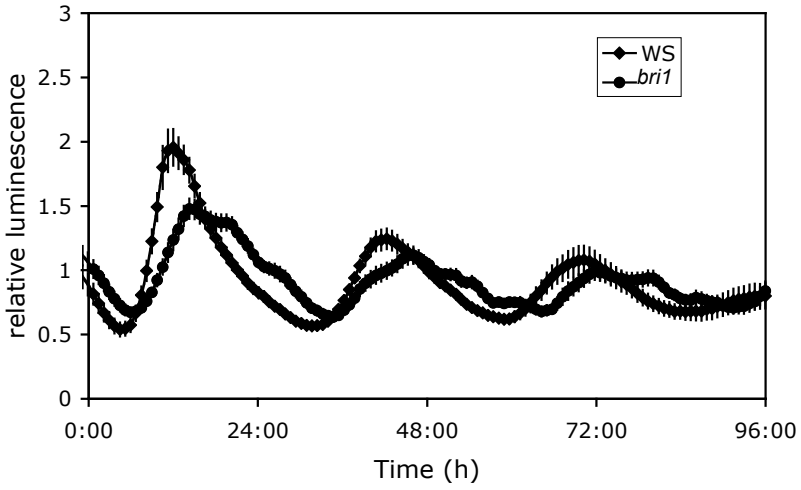

Figure 4.2. The circadian rhythms are altered in the *bri1* mutant. *CCR2::LUC* rhythms in *bri1* under constant light are shown. Note that the *bri1* mutation appears to lengthen periodicity.

Summary

A main developmental switch in the life cycle of a flowering plant is the transition from vegetative to reproductive growth. To maximize reproductive success, the timing of the floral transition must be precisely controlled. In Arabidopsis, at least four genetic pathways, the photoperiod, the autonomous, the vernalization, and the gibberellin (GA) pathways, which integrate endogenous and environmental signals, regulate the timing of this transition. A genetic screen was performed to identify additional components in the flowering-regulating network. This resulted in the isolation of two alleles of *bri1* as enhancers of the late-flowering phenotype of the autonomous mutant *luminidependens (ld)*. *BRI1* encodes an LRR-RLK (leucine-rich repeats receptor-like kinase) that functions as a receptor for brassinosteroids (BRs), thus the result of the screen indicated that *BRI1* or BRs could play a role in the floral timing. The aim of this thesis was to define and compare the roles of *BRI1* and BRs in floral transition. The studies were extended with examination of potential genetic interactions between BRs, GAs, and abscisic acid (ABA) in the control of the transition to the reproductive phase in Arabidopsis. To place *BRI1* in the flowering-genetic network, genetic and molecular-genetic approaches were used. Based on the analyses of various double mutants, which included combinations between *bri1* and known flowering-time mutants, it was concluded that the *BRI1* pathway has only a limited interaction with the photoperiod and the gibberellin pathways, and functions independently from vernalization. *BRI1* functions in part through the autonomous pathway. Synergistic interaction between the *BRI1* and the autonomous pathways was further confirmed by gene-expression studies. The *bri1* autonomous/*FRI* lines exhibited enhanced expression of the potent floral repressor *FLC,* which is known to be regulated by the autonomous and *FRI* pathways. The increased levels of *FLC* was accompanied by reduced expression of the downstream targets, *FT, SOC1, LFY*, whose expression is important for the floral transition to occur. Moreover, specific reduction of *FLC* via RNAi accelerated flowering of the *bri1 ld* double mutant. Based on the presented results, a model was proposed that describes the *BRI1* role in flowering-time control, where *BRI1* promotes flowering by genetically interacting with the autonomous pathway to repress the potent floral repressor *FLC*. To define the relationship between *BRI1* and BRs, the flowering-time phenotypes of the *bri1* mutant and a BR-biosynthesis mutant *cpd* were compared. It could be inferred from these studies that the BR pathway also interacts with the autonomous pathway, and the *bri1* phenotype could be partly explained by the BR-deficiency. However, due to differences in the flowering behavior of *cpd* and *bri1*, it was proposed that *BRI1* also exerts its function through unknown *BRI1*-specific factors. The plausible mechanisms explaining additional flowering phenotypes of the *bri1* were discussed. The role of BRs in the control of flowering time was also studied in the context of its possible interactions with the GA- and ABA-regulated pathways. The analyses of flowering phenotypes of double mutant combination deficient in BRs, ABA, GA did not reveal strong genetic interactions. The expression studies of key flowering-time genes in single and double hormone mutant combinations supported the model where the balance in the levels of these three hormones is necessary for appropriate timing of floral transition. Furthermore, based on the mild phenotype of the BR/ABA-deficient

mutants, and the flowering behaviors of transgenic lines that overexpress *DWF4, NCED3,* and *GA5,* leading to BR-, ABA- and GA- overproducing phenotypes, respectively, it has been concluded that GA has a limiting, and BR, ABA have the supporting function in the control of flowering time in Arabidopsis. Attempts were made to verify whether the BR-pathway converge on the promoter of *LFY*, which is one of the floral-pathways integrators. For this purpose, the *LFY::LUC+* reporter system was constructed, validated, and tested for BR-induction. No clear activation of the *LFY* promoter in the BR-overproducing lines was observed. In summary, the results described here provide evidence that both the BR-receptor BRI1 and brassinosteroids are important factors of floral-regulating network in Arabidopsis. Surprisingly, however, *BRI1* exerts its effects on flowering only partially through the BR-regulated pathway. The nature of *BRI1*-specific effects on flowering time remains to be investigated.

Zusammenfassung

Der wichtigste Entwicklungsschritt im Lebenszyklus einer Blühpflanze ist der Übergang von vegetativem zu reproduktivem Wachstum. Zur Maximierung des Reproduktionserfolges muss der Zeitpunkt dieses Übergangs präzise kontrolliert werden. In Arabidopsis regulieren mindestens vier genetische Signalwege, der photoperiodische Weg, der autonome Weg, die Vernalisierung und der Gibberellin-Weg, die jeweils endogene Signale und Umweltsignale integrieren, den Zeitpunkt des Übergangs. Zur Identifikation weiterer Komponenten des Kontrollnetzwerkes des Blühzeitpunktes wurde eine genetische Analyse durchgeführt. Dabei wurden zwei Allele von *bri1* als Verstärker eines spätblühenden Phänotyps der Autonom-Mutante *luminidependens* (*ld*) identifiziert. *BRI1* kodiert für LRR-RLK (leucine rich repeat – Receptor like kinase), einem Rezeptor für Brassinosteroide (BRs). Das Ergebnis der Analyse deutete auf die Beteiligung von *BRI1* oder BRs an der zeitlichen Kontrolle des Blühzeitpunkts hin. Ziel der vorliegenden Arbeit war es daher, den Einfluss von *BRI1* und BRs auf den Blühzeitpunkt zu definieren und zu vergleichen. Erweitert wurde die Arbeit durch die Untersuchung möglicher genetischer Interaktionen von BRs, Gibberellinsäure (GA) und Abscisinsäure (ABA) bei der Kontrolle des Übergangs von vegetativer zu reproduktiver Phase in Arabidopsis. Um die Position von *BRI1* im genetischen Netzwerk des Blühzeitpunkts zu bestimmen, wurden genetische und molekularbiologische Ansätze verwendet. Aus der Analyse verschiedener Doppelmutanten, einschließlich der Kombinationen von *bri1* mit bereits bekannten Blühzeitpunkt-Mutanten, konnte geschlossen werden, dass der *BRI1*-Signalweg nur geringe Wechselwirkungen mit dem photoperiodischen und dem Gibberellin-Weg hat und von der Vernalisierung unabhängig ist. Die Wirkung von *BRI1* entfaltet sich teilweise im autonomen Signalweg. Die synergistische Wechselwirkung von *BRI1* und dem autonomen Weg wurde durch Genexpressionsstudien bestätigt. Die *bri1* autonom/*FRI* Linien zeigte eine erhöhte Expression des starken Blüh-Repressors *FLC*, der durch den autonomen und den *FRI* Signalweg reguliert wird. Die erhöhte *FLC*-Konzentration wurde von einer reduzierten Expression seiner nachgeordneten Targets *FT, SOC1* und *LFY* begleitet, deren Expression für den Übergang in die Blühphase wichtig ist. Ferner beschleunigte die spezifische Reduktion von *FLC* durch RNAi den Blühzeitpunkt der *bri1 ld* Doppelmutante. Basierend auf den gezeigten Ergebnissen wurde ein Modell zur Rolle von *BRI1* in der Kontrolle des Blühzeitpunktes entwickelt, in dem *BRI1* den Blühzeitpunkt durch die Wechselwirkung mit dem autonomen Signalweg und der Unterdrückung des starken Blührepressors *FLC* indirekt fördert. Zur Bestimmung der Wechselwirkung von *BRI1* und BRs wurden die Phänotypen der Blühzeitmutante *bri1* und BR-biosynthese Mutanten *cpd* verglichen. Daruas ergab sich, dass der BR Signalweg mit dem autonomen Signalweg interagiert und dass der *bri1* Phänotyp teilweise durch eine BR-Defizienz erklärt werden kann. Die Unterschiede im Blühverhalten von *cpd* und *bri1* deuten jedoch darauf hin, dass *BRI1* seine Wirkung auch durch bislang unbekannte *BRI1*-spezifische Interaktoren ausübt. Mögliche Erklärungen für die verschiedenen Phänotypen werden diskutiert. Die Rolle von BRs in der Kontrolle des Blühzeitpunktes wurde auch im Hinblick auf mögliche Interaktionen mit den GA- und ABA-regulierten Signalwegen untersucht. Die Analyse von Blüh-Phänotypen verschiedener Kombinationen von Doppelmutanten mit Defizienzen bei BRs, ABA oder GA ergab keine starken genetischen

Wechselwirkungen. Expressionsstudien der Schlüsselgene des Blühzeitpunktes in diesen Einfach- und Doppelhormonmutanten unterstützte die These, nach der die Balance der Konzentration dieser drei Hormone für die Kontrolle des Blühzeitpunktes notwendig ist. Aus dem milden Phänotyp der BR/ABA-defizienten Mutanten und dem Blühverhalten der transgenen Überexpressionslinien *DWF4, NCED3* und *GA5,* die BR, ABA und GA überproduzieren, wurde geschlossen, dass GA einen limitierenden Effekt und BR und ABA eine unterstützende Funktion in der Kontrolle des Blühzeitpunktes in Arabidopsis haben. Weitere Untersuchungen sollten klären, ob der BR-Signalweg den *LFY* Promoter als einer der wesentlichen Integratoren der Kontrolle des Blühzeitpunkts aktiviert. Dazu wurde ein *LFY:LUC+* Reporter-System konstruiert, validiert und auf die Induktion von BR getestet. Es wurde jedoch keine klare Aktivierung des *LFY* promoters in BR-überproduzierenden Linien beobachtet. Zusammenfassend bestätigen die Ergebnisse, dass sowohl der BR-Rezeptor *BRI1* wie auch Brassinosteroide selbst wichtige Faktoren im Netzwerk der Blühzeitpunkt-Regulation in Arabidopsis sind. Erstaunlicherweise entfaltet *BRI1* seine Wirkung auf das Blüherhalten nur teilweise durch den BR-regulierten Signalweg. Der Charakter der spezifischen Effekte von BR bleibt daher noch zu untersuchen.

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Appendix

Erklärung

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

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