Genomic and Genetic Studies of Environmental Control of Brachypodium Growth and Development

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Genomic and genetic studies of environmental control of *Brachypodium* growth and development

by

Ying Feng

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Shuizhang Fei, Co-Major Professor
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Ames, Iowa
2013

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CHAPTER 1. GENERAL INTRODUCTION

*Brachypodium distachyon* has recently been established as a model for cereals and temperate grasses because of its sequenced small genome (~270M), a small stature, short life cycle, self-fertility, an efficient transformation system and particularly its diverse ecotypes (Brkljacic et al., 2011; Draper et al., 2001; Vogel et al., 2010). Compare to rice, *Brachypodium* has closer phylogenetic relationships with temperate grasses, moreover, it also has some unique biological features such as the ability to cold acclimate and its requirement of vernalization for flowering. Currently genome and transcriptome sequence are available for *Brachypodium*. Determination of the function for each gene in the *Brachypodium* genome is urgently needed for utilization of this species.

Flowering is a critical growth transition from vegetative to reproductive growth in plants. In plants with a winter growth habit, an exposure to a period of low temperature is essential to induce flowering, a process known as vernalization (Baurle and Dean, 2006; Boss et al., 2004). The molecular mechanism of vernalization-induced flowering has been extensively studied in *Arabidopsis*, wheat and barley (Amasino, 2005; Distelfeld et al., 2009; Izawa, 2007; Schmitz and Amasino, 2007; Srikanth and Schmid, 2011). However, comparative studies showed that the vernalization pathway is not conserved between *Arabidopsis* and temperate cereals (Amasino, 2005; Andres and Coupland, 2012; Dennis and Peacock, 2009; Distelfeld et al., 2009; Greenup et al., 2009; Kim et al., 2009; Trevaskis et al., 2007). Moreover, the findings of vernalization
pathway in temperate cereals are quite not consistent, so there still need more evidences to elucidate the vernalization pathway in monocots.

In general, plants with a winter growth habit (vernalization requiring) have better freezing tolerance compared to plants with a spring growth habit (non-vernalization requiring) before the onset of the reproductive growth phase, suggesting a link between vernalization requirement and freezing tolerance (Antikainen and Griffith, 1997; Chawade et al., 2012; Limin and Fowler, 2006; Sandve et al., 2011). But how vernalization is related to freezing tolerance remains unclear. Previous findings showed that vernalization pathway and cold acclimation pathway are interconnected (Dhillon et al., 2010; Lee et al., 2012; Seo et al., 2009). Cold acclimation is a short-term low but nonfreezing temperature treatment to plant, which induce the responsive genes C-repeat binding factor (CBFs) and Cold Regulated (COR) to increase the freezing tolerance of plant (Chinnusamy et al., 2007; Chinnusamy et al., 2010; Thomashow, 1999).

RNA interference (RNAi) is a powerful tool to determine gene function through the generation of loss-of-function mutants. In plants, the most effective gene silencing strategy is to use hairpin RNA (hpRNA) constructs containing inverted repeats (IRs), which generate siRNAs (small interfering RNA) that silence target genes based on sequence homology (Waterhouse et al., 1998; Waterhouse and Helliwell, 2003). To generate a plant mutant population with the majority of the genes in the genome silenced, one would construct many distinct RNAi constructs, so a high throughput approach for making hpRNA constructs is in great demand. Cell- or tissue- specific, or stress- specific cDNA populations would allow mutagenesis of a subset of genes
preferentially over other genes in the genome; thereby increasing the chance of a gene of interest being silenced and subsequently isolated. So a cold-specific RNAi library will facilitate to detect the functions of genes involved in cold response or vernalization pathway.

Brassinosteroids (BRs) play diverse roles in plant growth, development, and responses to different environmental factors. Previous studies found that application of exogenous BL affects cell expansion, cell division, vascular differentiation, male sterility, senescence, and modulation of stress responses (Clouse and Sasse, 1998; Divi and Krishna, 2009; Mandava, 1988). It is reported that manipulation of the BR signaling genes can improve plant stress tolerances (Divi and Krishna, 2009; Kim et al., 2010; Koh et al., 2007; Yang et al., 2010). However, the mechanism of BR signaling on stress tolerance and how BR signaling components regulate the induction of the stress-responsive genes remains to be addressed. Brassinosteroids receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) is a transmembrane serine/threonine kinase receptor with an extracellular domain containing leucine-rich repeats (LRRs) and an island domain that binds BR (Clouse et al., 1996; He et al., 2000; Hothorn et al., 2011; Kinoshita et al., 2005; Li and Chory, 1997; She et al., 2011; Wang et al., 2001), which has not been reported in Brachypodium.

The objectives of our studies are to: (1) analyze the expression of each gene involved in vernalization pathway in Brachypodium and the correlation of vernalization requirement with freezing tolerance, (2) develop a novel high-throughput RNAi method, Phi29-Amplified RNAi Construct (PARC), to construct a cold-specific RNA
interference library, for detecting the mechanism of cold acclimation and how plants develop freezing tolerance in *Brachypodium*, (3) knockdown brassinosteroids receptor gene *BRI1* by PARC in *Brachypodium* to characterize the loss-of-function *BdBRI1*-RNAi mutants. These studies will shed light on the pathways involved in vernalization, cold acclimation, freezing tolerance, brassinosteroids signaling, and drought tolerance. PARC can either be use to generate a high-throughput long hairpin (lhRNA) expression library or a lhRNA construct of a single gene.

**Dissertation organization**

The dissertation is organized in the format consisting of three journal-styled manuscripts accompanied by a general introduction and a general conclusion. The manuscripts are formatted according to each targeted journal. The first manuscript “Determination of the role of *VRN* genes in vernalization requirement and freezing tolerance in *Brachypodium distachyon*” will be submitted for publication in the *Journal of Experimental Botany*. I conducted all the experimental works and am the primary investigator for this work under the supervision of Drs. Shuizhang Fei and Yanhai Yin. The second manuscript “Construction of a high throughput cold-responsive specific RNAi library for the model grass *Brachypodium distachyon*” will be submitted for publication in *The Plant Journal*. This work is presented in Chapter 3 as a joint paper with Drs Jiangli Dong, Conglie Ma, and Richard Jorgensen. Dr Jiangli Dong did the primary experimental work for stem-loop rescue. Dr Conglie Ma and Richard Jorgensen proposed the PARC method for RNAi library construction. I was responsible for the
works of cDNA and RNAi library construction under the supervision of Drs. Shuizhang Fei and Yanhai Yin. The third manuscript “Down-regulation of *BdBRI1*, a *BRI1* homolog from *Brachypodium distachyon*, enhances drought tolerance” will be submitted to *New Phytologist*. I conducted all the experimental works and am the primary investigator for this work under the supervision of Drs. Shuizhang Fei and Yanhai Yin.

**References**


CHAPTER 2 DETERMINATION OF THE ROLE OF VRN GENES IN VERNALIZATION REQUIREMENT AND FREEZING TOLERANCE IN BRACHYPODIUM DISTACHYON

A paper to be submitted to Journal of Experimental Botany

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Abstract

Vernalization is an essential process to induce flowering for many temperate plant species. Brachypodium distachyon is a newly developed model plant for temperate grasses, in which vernalization requirement for flowering has not been characterized yet. Here, we isolated three vernalization homologue genes of BdVRN1, BdVRN2 and BdVRN3 from Brachypodium, and analyzed the expression of each gene in an accession that is either non-vernalization requiring (Bd21), or vernalization requiring (Bd29-1)
growth habit. We found that vernalization induced expression of *BdVRN1* and *BdVRN3* is required for inflorescence development in Bd29-1, but not in Bd21. Moreover, vernalization induced expression of *BdVRN1* precedes that of *BdVRN3*. RNAi knockdown mutants for *BdVRN1* conferred vernalization requirement for flowering in Bd21, and resulted in reduced expression of *BdVRN3*, but not *BdVRN2*. When subjected to freezing treatment, both Bd29-1 and the *BdVRN1* RNAi mutants of Bd21 exhibited reduced freezing tolerance after vernalization, suggesting a negative correlation between expression of *BdVRN1* and freezing tolerance. Without vernalization, several cold responsive genes *BdCBF2*, *BdCBF3*, *BdCBF5*, *BdCBF6*, and *BdDREB2A* were all constitutively expressed at a high level in the *BdVRN1* RNAi mutants of Bd21. Taken together, these results suggest that expression of *BdVRN1* promotes flowering by upregulation of *BdVRN3* in the vernalization pathway, and at the same time reduces freezing tolerance by downregulation of cold responsive genes.

**Key words:** vernalization, freezing tolerance, cold acclimation, flowering.

*Brachypodium*
Introduction

Flowering is a critical growth transition from vegetative to reproductive growth in plants. In plants with a winter growth habit, an exposure to a period of low temperature is essential to induce flowering, a process known as vernalization (Baurle and Dean, 2006; Boss et al., 2004). On one hand, vernalization prepares plants to acquire the competence to flower; on the other hand, vernalization postpones the flowering time of plants to prevent the cold-sensitive flowering meristem from being damaged during the winter (Balasubramanian et al., 2006; Baurle and Dean, 2006; Kim et al., 2004; Trevaskis et al., 2007).

The molecular mechanism of vernalization-induced flowering has been extensively studied in Arabidopsis, wheat and barley (Amasino, 2005; Distelfeld et al., 2009; Izawa, 2007; Schmitz and Amasino, 2007; Srikanth and Schmid, 2011).

FLOWERING LOCUS T (FT) in Arabidopsis is identified to be the “florigen” (Kardailsky et al., 1999; Kobayashi et al., 1999), which encodes a small globular protein that is able to translocate from the leaves to the shoot apex through the phloem, where it interacts with a bZIP transcription factor FLOWERING LOCUS D (FD) to activate the floral meristem identity gene APETALAI (API) for flowering (Abe et al., 2005; Wigge et al., 2005). In wheat and barley, VERNALIZATION 3 (VRN3) and VERNALIZATION 1 (VRN1) have been identified to be the homologues of the Arabidopsis FT and API, respectively (Danylyuk et al., 2003; Faure et al., 2007; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2006; Yan et al., 2003).
However, comparative studies showed that the vernalization pathway is not conserved between *Arabidopsis* and temperate cereals (Amasino, 2005; Andres and Coupland, 2012; Dennis and Peacock, 2009; Distelfeld *et al.*, 2009; Greenup *et al.*, 2009; Kim *et al.*, 2009; Trevaskis *et al.*, 2007). In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*), a MADS-box transcript factor, has been identified to be a negative regulator of flowering, which blocks the long-day induction of *FT* but itself is repressed by vernalization (Michaels and Amasino, 1999; Searle *et al.*, 2006). In temperate cereals, no homologous gene of *FLC* is found, but *VERNALIZATION 2* (*VRN2*) gene was identified as a flowering repressor in cereals, which encodes a zinc-finger and CCT (CONSTANS, CONSTANS-LIKE, TOC1) domain protein (ZCCT1) (Yan *et al.*, 2004). *VRN2* is induced by long-day and can function like *FLC* to suppress long-day induction of *VRN3* (Yan *et al.*, 2006; Yan *et al.*, 2004). *VRN2* also has been reported to repress *VRN1* gene, but this repression is relieved by vernalization (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). *VRN1* is induced by vernalization further to repress *VRN2*, thereby releasing the repression of *VRN3* by *VRN2* (Yan *et al.*, 2006; Yan *et al.*, 2004; Yan *et al.*, 2003). And then, *VRN3* further elevates the expression of *VRN1* to induce flowering (Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006; Yan *et al.*, 2004). These interactions form a feedback loop leading to flowering based on *VRN2-VRN3 (FT)-VRN1* model in wheat.

Shimada *et al.* (2009) proposed an alternative model of vernalization response in wheat. In their model, *VRN1* is thought to be upstream of *VRN1* and up-regulates the *VRN3* expression under vernalization treatment, which is opposite to the first model. The
up-regulation of VRN3 further represses VRN2, releasing the inhibition of VRN1 by VRN2, also in opposition to the first model (Shimada et al., 2009). Therefore, the interaction among these three genes still needs to be clarified.

In general, plants with a winter growth habit (vernalization requiring) have better freezing tolerance compared to plants with a spring growth habit (non-vernalization requiring) before the onset of the reproductive growth phase, suggesting a link between vernalization requirement and freezing tolerance (Antikainen and Griffith, 1997; Chawade et al., 2012; Limin and Fowler, 2006; Sandve et al., 2011). But how vernalization is related to freezing tolerance remains unclear. Previous findings showed that vernalization pathway and cold acclimation pathway are interconnected (Dhillon et al., 2010; Lee et al., 2012; Seo et al., 2009). 

**SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1),** encoding a MADS-box transcription factor, has been reported to regulate multiple floral induction pathways including vernalization, photoperiod and autonomous (Onouchi et al., 2000; Samach et al., 2000). **SOC1** may also play an important role on regulation of cold acclimation, a short-term exposure to low but nonfreezing temperatures. Cold acclimation induces the cold responsive genes including the **C-repeat binding factor (CBFs)** and **Cold Regulated (COR)** to increase the freezing tolerance of plants (Chinnusamy et al., 2007; Chinnusamy et al., 2010; Thomashow, 1999). The knockout and overexpression mutants of **SOC1** either increased or decreased the expression of cold responsive genes such as **CBFs** and **COR**, respectively (Seo et al., 2009). In addition, heterologous expression of the wheat **VRN2 (TaVRN2)** gene in **Arabidopsis** delayed flowering and enhanced freezing tolerance due to the accumulation
of $CBF_2$, $CBF_3$ and $COR$ genes (Diallo et al., 2010). In wheat and barley, a locus designated $FR-2$ was associated with freezing tolerance (Francia et al., 2004; Galiba et al., 2009; Vagujfalvi et al., 2003). Molecular characterization revealed that the $FR-2$ locus harbors a group of $CBF$ genes (Francia et al., 2007; Knox et al., 2010; Miller et al., 2006). Some evidences showed that $VRN1$ has a negative effect on the expression of $CBFs$ at the $FR-2$ locus and further influencing freezing tolerance (Dhillon et al., 2010; Stockinger et al., 2007).

To elucidate the mechanism of vernalization pathway and its relationship with freezing tolerance, we used a recently established monocot model plant, *Brachypodium distachyon*. With its sequenced genome, a small physical stature, self-fertility, a short lifecycle, an efficient transformation system and particularly its diverse ecotypes, *Brachypodium* is well suited for studying vernalization and freezing tolerance.

In this study, we analyzed the expression of $VRN$ genes in *Brachypodium* of either non-vernalization requiring (Bd21) or vernalization requiring (Bd29-1) accession and studied their freezing tolerance. We found that $BdVRN1$ and $BdVRN3$ were induced by vernalization and were positive regulators for flowering. But $BdVRN2$ is likely not involved in the vernalization pathway. Knockdown of $BdVRN1$ in Bd21 led to a severe phenotype with delayed flowering, which could be rescued by vernalization treatment. Expression analysis of the $BdVRN1$ RNAi mutants showed that $BdVRN1$ positively correlated with the expression of $BdVRN3$ but had no effect on $BdVRN2$. Meanwhile, enhanced tolerance to freezing stress was observed in the RNAi mutants, accompanied by constitutive expression of several cold responsive genes, including $BdCBF2$, 
BdCBF3, BdCBF5, BdCBF6 and DREB2A at high levels. These results suggested that BdVRN1 plays a critical role on flowering in vernalization pathway, furthermore, acting as a negative signal affecting the regulation of the cold responsive genes.

Material and Methods

Plant materials and growth condition

Seeds of *B. distachyon* Bd21 (PI 254867) and Bd29-1 (PI 639818) were sown in each 6-inch pot containing Sun Gro Hort soil mix (Bellevue, WA) in the greenhouse at 25°C, 16/8 h (day/night) with an irradiance of 450 ± 30 μmol m⁻² s⁻¹.

Vernalization treatment

To determine the vernalization requirement for flowering, four-week-old plants of each accession were placed into a growth chamber at 4°C and 8/16 h (day/night) with an irradiance of 280 ± 30 μmol m⁻² s⁻¹ for 3, 6, 9, or 12 weeks. After vernalization they were moved back to a greenhouse (25°C, 16/8 h day/night) with an irradiance of 450 ± 30 μmol m⁻² s⁻¹.

To analyze the expression level of *BdVRN* genes, four-week-old plants of each accession were placed into a growth chamber at 4°C and 8/16 h (day/night) with an irradiance of 280 ± 30 μmol m⁻² s⁻¹ for 2, 4, 6, 8, 10 or 12 weeks. After vernalization they were moved back to a greenhouse (25°C, 16/8 h day/night) with an irradiance of 450 ± 30 μmol m⁻² s⁻¹ to initiate flowering.
RNAi vector construction and generation of RNAi mutants

A fragment of *BdVRN1* (414bp) gene were amplified by PCR (primers listed in Table S2) with the addition of four bases of CACC at its 3’ end. PCR was performed as follows: 94 °C for 5min; 35 cycles of 94 °C for 20 sec, 59 °C for 30 sec and 72 °C for 50 sec; final extension at 72°C for 1 min. The PCR product was separated on 1% agarose gel and extracted from the gel. The gene was cloned into the pENTR™/D- TOPO® vector (Life Technologies, Grand Island, NY ) containing attL recombination sites according to the manufacturer's protocol. The pANDA (Miki and Shimamoto, 2004) vector contains two cassettes in inverse orientation linked by a small fragment of the GUS gene. Kanamycin (*nptII*) and hygromycin (*hpt*) resistance genes were used for the selection in bacteria and plants, respectively. The attL × attR reaction is mediated by Gateway® LR Clonase™ II enzyme mix (Life Technologies, Grand Island, NY). The final binary vector pANDA::BdVRN1 was verified by sequencing.

The pANDA::BdVRN1 vector was introduced into Agrobacterium C58C1 strain following the protocol developed by Vogel and Hill (2008). The selection medium contained Timentin (bioWORLD, Dublin, OH) at 150 mg L⁻¹ to suppress *Agrobacterium* growth and selective agent hygromycin B (bioWORLD, Dublin, OH) at 40 mg L⁻¹ to kill untransformed calli for two cycles under dark at 23°C, each of which lasted two weeks. Resistant calli were moved into regeneration medium containing Kinetin (KT) at 1 mg L⁻¹ and hygromycin at 10 mg L⁻¹ in a tissue culture chamber at 23°C, 16/8h (light) with an irradiance of 180 ± 30 μmol m⁻² s⁻¹. Shoots started to appear after 7-10 days. Rooting took place on a MS medium supplemented with 0.1 mg L⁻¹ NAA and 10 mg L⁻¹.
hygromycin. Well-rooted plantlets were carefully moved into 6-inch pot containing Sun Gro Hort soil mix (Bellevue, WA). Plants were grown in a growth chamber at 23°C, 16/8h (day/night) with an irradiance of 400 ± 30 μmol m⁻² s⁻¹.

Transgenic plants were screened by PCR using primers (HPT-F 5’GAATTCAAGCGAGGCGCTG 3’, HPT-R 5’ ACATTGGTGAGCCGAAA 3’)) designed from the sequence of the hygromycin resistant gene present in the binary vector pANDA. Eight independent T₀ lines were confirmed by PCR analyses. In two of the independent lines, RNAi-4 and RNAi-12, PCR-positive and negative plants segregate in the progenies in a 3:1 ratio, suggesting the integration of the transgenes at a single locus in each of the two lines. The homozygous plants of T₂ RNAi-4 and RNAi-12 lines were chosen for further analyses.

**Assessment of freezing tolerance**

Freezing tolerance of Bd21, Bd29-1 and BdVRN/ RNAi mutants were assayed with or without vernalization or cold acclimation. For non-cold acclimation treatment, four-week old seedlings of each accession were either not vernalized or vernalized for 6 weeks at 4°C and 8/16 h (day/night) with an irradiance of 400 ± 30 μmol m⁻² s⁻¹ in a growth chamber. The vernalized plants were moved to a greenhouse (25°C, 16/8 h day/night) with an irradiance of 450 ± 30μmol m⁻² s⁻¹ for one week before the freezing treatment at -5°C and 8/16 h (day/night) 12 hours. Similarly, non-vernalyzed plants were grown for one week in the greenhouse (25°C, 16/8 h day/night) before the freezing treatment. Following the freezing treatment, plants were moved into a growth chamber
at 4°C for recovery for one day. And then they were placed back into a greenhouse (25°C, 16/8 h day/night) and were recorded for recovery one week later.

For cold acclimation treatment, the process was the same as stated above, except that the plants were cold acclimated at a 4°C and 16/8 h (day/night) with an irradiance of 400 ± 30 μmol m⁻² s⁻¹ in a cold chamber for 10 days, immediately followed by the aforementioned assessment of freezing tolerance.

**Semiquantitative RT-PCR analysis**

Total RNA was extracted from leaves or meristems with TRIzol® Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with the SuperScript® III First-strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA). PCR was performed with gene specific primers as follows: pre-denaturation at 94°C for 5 min; for 25 cycles of denaturation at 94°C for 30s, primer-annealing at 58°C for 30s, elongation at 72°C for 50s, and post elongation at 72°C for 5min. The primer sequences used are provided in the supplemental materials (Table S2). The expression level of each gene was normalized to that of the *BdGAPDH* gene.

**Real-Time RT-PCR analysis**

Total RNA was extracted with TRIzol® Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 5 μg total RNA by using the SuperScript® III First-strand Synthesis System (Invitrogen, Carlsbad, CA) and quantitative analyses were carried out on the Eco® Real-Time PCR System (Illumina, Inc., San Diego, CA) using
the SYBR® GreenER qPCR SuperMix™ kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity of PCR products was determined at the end of each cycle by the Eco® Software v4.0 (Illumina, Inc., San Diego, CA). The expression level of each gene was normalized to that of *BdGAPDH* gene, and the expression level for each gene in the wild type without treatment was set as 1.0. Primers used for PCR amplification are listed in the supplemental materials (Table S3).

**Results**

*Brachypodium* accession Bd29-1 required vernalization whereas accession Bd21 does not

Flowering time was determined for accessions Bd29-1 and Bd21 with or without vernalization treatment. Bd21 initiated flowering within 24 ± 3 days, independent of the vernalization treatment (Fig.1). Vernalization treatment had no effect on accelerating flowering in Bd21. In contrast, Bd29-1 did not flower within 140 days. When subjected to a 3-week vernalization treatment, Bd29-1 initiated flowering within 109 ± 6.2 d. When subjected to a 6-week vernalization treatment, Bd29-1 initiated flowering as early as 27 ± 4.2 days after vernalization. Longer vernalization treatment than 6 weeks had no further reduction in days to flowering. Therefore, accession Bd29-1 required vernalization treatment to initiate flowering, for which six weeks vernalization treatment may long enough.
Vernalization requirement of *Brachypodium* was closely associated with the expression of *BdVRN* genes

Three genes of *VRN1*, *VRN2* and *VRN3* (*FT*) that control the vernalization treatment in wheat or barley have been characterized. Recently, in silico comparative genomic analysis showed that *Brachypodium* has a *VRN1* homologue (*BdVRN1*), Bradi1g08340, located in a position colinear to the rice homologue (*OsMADS14*) and wheat homologue (*TaVRN-1*) genes, and an *VRN3* homologue gene Bradi1g48830. Although there was no *VRN2*-like gene in *Brachypodium*, Bradi3g10010 was identified to be an intermediate between *HvCO9* (a gene in photoperiod response for flowering) and *VRN2* in the phylogenetic tree (Higgins *et al.*, 2010). Therefore, we treat Bradi3g10010 as *BdVRN2*.

We next assayed the expression of the three genes (*BdVRN1*, *BdVRN2* and *BdVRN3*) in both the non-vernalization requiring (Bd21) and vernalization requiring (Bd29-1) types of *Brachypodium* with or without vernalization treatment. Without vernalization treatment, we found that expression of *BdVRN1* expression was kept at a low level in leaves or meristems during the first three weeks after germination in both Bd21 and Bd29-1 (Fig. 2A, B, C). After four weeks, it was increased by 20 folds in meristems in Bd21, whereas it remained unchanged in Bd29-1 (Fig. 2C). When subjected to vernalization treatment, expression of *BdVRN1* in meristems remained at the high level (with a slight decrease) in Bd21, whereas it was gradually increased to a similar level to that of Bd21 after 6 weeks of vernalization treatment in Bd29-1 (Fig. 2C).
BdVRN2 was detected with a high expression level in leaves (Fig. 2A) but was hardly detected in meristems both in Bd21 and Bd29-1 with or without vernalization (Fig. 2A, B, D). The expression of BdVRN2 in leaves and meristems were not influenced by vernalization in both Bd21 and Bd29-1 (Fig. 2B, D). In Bd21, expression of BdVRN3 increased continuously before vernalization treatment, and did not change noticeably by vernalization treatment (Fig. 2E). In contrast, in Bd29-1, the expression of BdVRN3 remained at a low level without vernalization, but it was raised by 6 folds after 8 weeks of vernalization (Fig. 2E).

The results showed that non-vernalization requiring and vernalization requiring Brachypodium had different expression profiles for BdVRN genes except for BdVRN2. Vernalization induced the expressions of BdVRN1 and BdVRN3 in both leaves and meristems in the vernalization requiring accession Bd29-1, but not in the non-vernalization requiring accession Bd21. In both accessions, BdVRN2 exhibited a similar expression pattern with or without vernalization treatment. These results suggested that the expression of BdVRN1 and BdVRN3 is related to the flowering time in Brachypodium with differred requirement for vernalization. For example, Bd21, in which both the putative floral activator BdVRN1 and BdVRN3 gene had a high expression level in meristems (Fig. 2A), could initiate flowering within 7 weeks following seed germination without any vernalization treatment (Fig. 1). In contrast, Bd29-1 in which neither BdVRN1 or BdVRN3 was sufficiently expressed (Fig. 2B, C, D) without vernalization treatment, could not initiate flowering even after 20 weeks (Fig. 1). As mentioned before, six weeks of vernalization was sufficient to fulfill the requirement of
vernalization for flowering in Bd29-1 (Fig. 1). Vernalization treatment significantly increased the expression levels of both \textit{BdVRN1} and \textit{BdVRN3} (Fig. 2C, E).

**Knockdown of \textit{BdVRN1} in Bd21 severely delayed flowering**

To determine whether \textit{BdVRN1} regulates flowering time, RNAi mutants of \textit{BdVRN1} were generated in the non-vernalization requiring accession Bd21. Eight independent T\textsubscript{0} transgenic lines were confirmed by PCR analysis and two of them, RNAi-4 and RNAi-12 exhibited a late flowering phenotype (Fig. 3A). Real-Time RT-PCR showed that the \textit{VRN1} transcripts were reduced to 0.12 ± 0.02 in RNAi-4 and 0.26 ± 0.04 in RNAi-12 (Fig. 3B). These two RNAi mutants did not flower within 140 days (Fig. 3C) without vernalization treatment. After a 6-week vernalization treatment, RNAi-4 and RNAi-12 mutants displayed delayed flowering, initiating flowering within 35.5 ± 5.6 d and 28.2 ± 8.3 d respectively, compared to 19.2 ± 5.4 d for wild type. The phenotypic change of Bd21 from non-vernalization requiring to vernalization requiring by knocking down \textit{BdVRN1} strongly suggests that \textit{BdVRN1} plays a critical role in flowering time through the vernalization pathway.

**Knockdown of \textit{BdVRN1} in Bd21 reduced the expression of \textit{BdVRN3}**

To further understand how \textit{BdVRN1} affects flowering time at the molecular level, we analyzed the expression patterns of \textit{BdVRN1}, \textit{BdVRN2} and \textit{BdVRN3} in the \textit{BdVRN1} knockdown mutants. The expression level of \textit{BdVRN1} was suppressed significantly in the 4-week old seedlings of RNAi mutants, but upregulated if treated with vernalization.
by 6 weeks (Fig. 4A). The expression of BdVRN2 was not affected in BdVRN1 RNAi mutant (Fig. 4B). Moreover, we found that the expression of BdVRN3 was intimately related to BdVRN1. When BdVRN1 was knockdown by RNAi, the expression level of BdVRN3 is decreased. When the expression of BdVRN1 was elevated by vernalization treatment, the expression level of BdVRN3 was increased (Fig. 4C). The results indicated that BdVRN1 transcripts have a positive correlation with BdVRN3, but not BdVRN2.

The vernalization requiring Brachypodium accession is more freezing tolerant than the non-vernalization requiring accession

To determine the relationship between the vernalization pathway and the freezing tolerance pathway, we compared the freezing tolerance of the vernalization requiring Bd29-1 and the non-vernalization requiring Bd21. Bd29-1 showed much higher recovery rate following a freeze than did Bd21 without a vernalization treatment (Fig. 5A). However, when plants were vernalized for 6 weeks, the recovery rate of Bd21 was unchanged whereas that of Bd29-1 decreased significantly (Fig. 5A). With cold acclimation, both Bd21 and Bd29-1 had an increase in recovery rate compared with their non-cold acclimated controls (Fig. 5B). The result that vernalization treatment reduced the freezing tolerance ability of Bd29-1 but not Bd21 suggested that vernalization pathway may be negatively correlated with plants freezing tolerance.
BdVRN1 functions as a negative regulator of the freezing tolerance pathway in Brachypodium

In order to determine a possible connection between the enhanced freezing tolerance and vernalization requirement, we compared the freezing tolerance of the wild type Bd21 with that of the BdVRN1 RNAi mutants, which showed suppressed BdVRN1 expression and resemblance to Bd29-1 in vernalization requirement. We found that the freezing tolerance of the RNAi mutant was similar to that of Bd29-1 (Fig. 5A, B), suggesting that the enhanced freezing tolerance of Bd29-1 and the Bd21 RNAi mutants was associated with their low BdVRN1 expression levels. Semiquantitative RT-PCR experiments confirmed that Bd29-1 and Bd21 RNAi mutants had lower BdVRN1 expression levels than the wild type Bd21 without vernalization treatment, while a 6-week vernalization treatment increased the expression levels of BdVRN1 in both accessions to a similar level in Bd21 (Fig. 5C).

To further characterize the relationship between BdVRN1 gene and freezing tolerance, we analyzed the expression of cold-responsive genes by real-time RT-PCR in both the wild type Bd21 and its RNAi mutants without vernalization treatment. CBF genes are induced by cold stress and regulate other cold stress responsive genes expression for increased freezing tolerance (Catala et al., 2003; Dubouzet et al., 2003; Ito et al., 2006; Jaglo-Ottosen et al., 1998; Lata and Prasad, 2011; Liu et al., 1998; Xiong and Fei, 2006). For example, CBF1 in Arabidopsis is the transcriptional activator for the COR genes, such as COR47 and COR414-TM1, which are involved in cold and dehydration pathway (Griffith et al., 2007; Sakuma et al., 2006; Xiong et al., 2002).
DREB2A is a transcription factor induced by dehydration in Arabidopsis, rice, soybean and maize (Cui et al., 2011; Mizoi et al., 2013; Qin et al., 2007; Sakuma et al., 2006; Simpson et al., 2003). The RD (RESPONSIVE TO DESICCATION) genes, for example RD26 and RD29B, are induced by cold or drought stress through the regulation of the transcription factor DREBs (Msanne et al., 2011; Nakashima et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 1994).

In a previous study, we isolated eight CBF genes (CBF1- CBF8) from Brachypodium that are all cold induced (data to be reported elsewhere). Here we compared their expressions with other important cold responsive genes between the wild type Bd21 and its BdVRN1 RNAi mutants. We showed that CBF2, CBF3, CBF5, CBF6, COR414-TM1 and COR47 were highly induced from 3 to 600 folds in both the wild type and the RNAi mutants after 24 hours of cold treatment (Fig. 6A). More interestingly, CBF2, CBF3, CBF5, CBF6 and DREB2A were constitutively expressed at high levels in the RNAi mutants without cold treatment, and the constitutive expression of CBF3 and DREB2A in the RNAi mutant was even higher than that of the cold-treated wild type. On the other hand, RD26 and RD29B genes showed no noticeable changes in the RNAi mutant, compared to the wild type. When BdVRN1 expression level was increased by vernalization, expression levels of those genes that were constitutively expressed in the RNAi mutants at high levels were reduced (Fig. 6B). Specifically, expression levels of CBF3 and DREB2A were decreased to the similar level as that in the wild type after vernalization treatment. These results indicated that BdVRN1 suppresses expression of
several important cold-responsive genes, and therefore plays an important role in connecting the vernalization pathway with the cold response pathway.

Discussion

In this study, we assayed the requirement for vernalization in accessions Bd29-1 and Bd21, of which the genome has been sequenced. We found that Bd29-1 requires vernalization to induce flowering but the flowering of Bd21 is independent of vernalization treatment. To understand the molecular basis of the differences in vernalization requirement between the two accessions, we isolated three homologues of known vernalization genes, *BdVRN1*, *BdVRN2* and *BdVRN3* from both accessions, and analyzed their expression. We found that the vernalization requirement is closely correlated with the expression pattern of the *BdVRN1* and *BdVRN3* genes, but not the *BdVRN2* gene. Bd21 initiated flowering in 7 weeks following seed germination, accompanied by high levels of expression of both *BdVRN1* and *BdVRN3* independent of a vernalization treatment. By contrast, Bd29-1 required 6 weeks of vernalization to induce the expression of *BdVRN1* and *BdVRN3* and to promote flowering effectively. When subjected to longer vernalization treatment, the expression of *BdVRN1* was increased to a higher level, whereas the expression of *BdVRN3* was decreased gradually. Additionally, the induction of *BdVRN1* expression was earlier than *BdVRN3* in Bd29-1. However, the expression of *BdVRN2* was not correlated to vernalization treatment in both Bd21 and Bd29-1. Therefore, we speculated that *BdVRN1* and *BdVRN3* positively regulate vernalization response of the vernalization requiring accession Bd29-1. To
further characterize the vernalization pathway in *Brachypodium*, we developed *BdVRN1* RNAi mutants of Bd21. The loss-of-function mutants exhibited vernalization-requiring phenotype, similar to Bd29-1. Gene expression analysis showed that the expression of not only *BdVRN1* but also *BdVRN3* were suppressed in the mutants, however expression of both were stimulated by vernalization treatment. The expression of *BdVRN2* was not affected in the RNAi mutants with or without vernalization treatment, suggesting *BdVRN2* may not be a true homologue, or is not involved in flowering in *Brachypodium*.

Our result is consistent with previous studies in wheat and barley that both *VRN1* and *VRN3* are induced by vernalization with a quantitative effect on the timing of flowering initiation (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Yan *et al.*, 2006; Yan *et al.*, 2003). Different hypotheses have been proposed on the relationship between *VRN1* and *VRN3* in temperate cereals. Li and Dubcovsky (2008) thought *VRN3* regulate the *VRN1* expression in the vernalization pathway, while others suggested that *VRN1* is upstream of *VRN3* (Sasani *et al.*, 2009; Shimada *et al.*, 2009). Our results largely agree with the idea that *VRN1* is induced by vernalization and upregulates the expression of *VRN3*.

Comparative genomic analysis suggested that *VRN2* gene may be deleted in the Bd21 genome, similar to rice, which has no requirement for vernalization (Higgins *et al.*, 2010). In our work, we chose a *CCT* gene grouped between *HvCO9* and *VRN2* in a phylogenetic tree as *BdVRN2*. We showed that *BdVRN2* was stably expressed in Bd21, which is consistent with the idea by comparative genomic analysis that this is a loss-of-function *BdVRN2* in Bd21. However, the *BdVRN2* was also stably expressed
independent of the vernalization both in Bd29-1 and the BdVRN1 RNAi mutants of Bd21, suggesting that BdVRN2 is not involved in the vernalization pathway. This is different from wheat or barley, in which VRN2 was characterized as a repressor of flowering and is regulated by both photoperiod and vernalization. Our result suggested that there are evolutionary difference between Brachypodium and temperate cereals.

It is well observed that vernalization requiring plants have better freezing tolerance than the non-vernalization requiring plants, but the molecular link between them remains unclear. Previous works suggested that there might be a negative correlation between freezing tolerance and VRN1 transcript in wheat (Danyluk et al., 2003; Limin and Fowler, 2006). The expression of TaVRT-1, a homologue of AP1 in wheat, was positively associated with vernalization, and was negatively associated to the accumulation of COR genes and freezing tolerance (Danyluk et al., 2003). Expression studies showed that high levels of constitutive expression of some CBF genes in wheat winter cultivars gave a higher freezing tolerance compared to spring cultivars (Badawi et al., 2007; Kobayashi et al., 2005). In addition, the VRN1 deletion mutant, mvp (maintained vegetative phase), increased freezing tolerance along with an increased expression level of several CBF and COR genes (Dhillon et al., 2010). A subsequent study found that the deletion of the mvp mutant include other genes besides VRN1 such as AGL1, CYS, PHYC (Distelfeld and Dubcovsky, 2010). In the present study we showed that the vernalization requiring Bd29-1 accession is more freezing tolerant than the non-vernalization requiring Bd21, which corresponds to the difference of transcript levels of BdVRN1 between the two accessions. Significantly, when we treated the Bd29-
1 and Bd21 with vernalization for 6 weeks, the freezing tolerance of Bd29-1 was reduced, but that of Bd21 remained the same. In order to eliminate other factors in regulating freezing tolerance, we compared the freezing tolerance between the non-vernalization requiring Bd21 and its BdVRN1 RNAi mutants. Our results showed that freezing tolerance was enhanced in BdVRN1 knockdown mutants relative to the wild type. Vernalization treatment of the RNAi mutants reduced their freezing tolerance. Moreover, several key cold-responsive genes, BdCBF2, BdCBF3, BdCBF5, BdCBF6 and DREB2A, are constitutively expressed at high levels in the knockdown mutants. Whether BdVRN1 directly regulates freezing tolerance pathway or function corporately with other genes remains to be determined.

Flowering induction is an important and complicate process, which requires a series of genes to function coordinately. In nature, temperate plants need to go through the winter to induce flowering and to generate seeds. On one hand, VRN1 transcripts are maintained at a low level before the winter, signaling that winter has not passed yet, so plants need to maintain a high level of expression for the cold- responsive genes to defend plants from freezing damage. Once the VRN1 transcript level has reached the threshold of acquiring flowering competency following a long winter, it signals that winter has passed and the plants make the transition from vegetative phase to reproductive phase and decrease the expression of cold- responsive genes in order to save energy for reproductive use.

In summary, our work characterized the vernalization response and its relationship with freezing tolerance in Brachypodium by a comparative study of a non-
vernalization requiring accession Bd21 with a vernalization requiring accession Bd29-1 and the \( \text{BdVRN1} \) RNAi mutants of Bd21. We found that \( \text{VRN1} \) is regulated by vernalization in the vernalization requiring Bd29-1, which is able to induce the expression of \( \text{BdVRN3} \) to cooperatively stimulate flowering with vernalization and function as a negative regulator of several key cold-responsive genes. Whether any repressor of vernalization for flowering is existed in \( \text{Brachypodium} \) winter type Bd29-1 is still not clear, and the detail mechanism of \( \text{VRN1} \) regulation of cold-responses still needs to be addressed.

Acknowledgments

We thank John Vogel at USDA-ARS for kindly providing seeds of \( \text{Brachypodium distachyon} \). We acknowledge from the Plant Science Institute at Iowa State University, the Consortium for Plant Biotechnology Research Inc (GO12026- 322 to S.F and Y.Y) and the United States Golf Association.

References


Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-


Tables

Table S1. Results of BLAST search of vernalization-related genes and cold stress inducible genes in *B. distachyon*

<table>
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<th>Gene</th>
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BLAST searches of *B. distachyon* genome were carried on Phytozome V9.0 server (http://www.phytozome.com/search.php?show=text&method=Org_Bdistachyon) using the query amino acid sequences. The putative genes were isolated with primers designed from the coding region of each gene (Table S2).
Table S2. Sequences of primers used for Semiquantitative RT-PCR

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Table S3. Sequences of primers used for Real-Time RT-PCR

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Fig. 1 Average number of days required for flowering for the non-vernalization requiring accession Bd21 and the vernalization requiring accession Bd29-1 after vernalization treatment. Four-week old seedlings of each accession were either without vernalization or vernalized for 3, 6, 9, or 12 weeks at 4°C and 8/16 h (day/night) in a growth chamber. After vernalization treatment, plants were transferred to a greenhouse (25°C, 16/8 h day/night). NF denotes no flowering observed. Average number of days to flowering indicated the number of days required for flowering following vernalization treatment. Error bars represent the standard deviation from independent plants.

Fig. 2 Expression analysis of BdVRN genes in leaves and meristems in Bd21 (non-vernalization requiring) and Bd29-1 (vernalization requiring). A. Semiquantitative PCR of BdVRN genes in leaves of 2, 3, or 4-week old seedlings and in meristems of 4-week old seedlings from Bd21. B. BdVRN genes expression in leaves and meristems from Bd29-1, 4-week old seedlings were vernalized for 2, 4, 6, 8, 10, and 12 weeks (BdGAPDH was used as loading control). C-E. Relative expression levels of BdVRN1, BdVRN2 and BdVRN3 genes in meristems of Bd21 and Bd29-1. Before vernalization, plants were grown at a green house (25°C, 16/8 h day/night). Samples were collected from 2, 3, 4-week old seedlings. Four-week old seedlings then were transferred to a cold growth chamber at 4°C and 8/16 h (day/night). Meristems were collected after 2, 4, 6, 8, 10, or 12 weeks of vernalization treatment.
Fig. 3 A. Wild type (left) and BdVRN1 RNAi mutant plant (right) 6 weeks after seed germination. B. Real-Time RT-PCR analysis of the BdVRN1 expression level in the wild type (WT), knock down mutants of BdVRN1 RNAi-4 and RNAi-12. C. Average number of days required for flowering for the wild type Bd21 and RNAi mutants after vernalization treatment. Four-week old seedlings of each accession were either not vernalized or vernalized for 6 weeks at 4°C and 8/16 h (day/night) in a growth chamber. After the vernalization treatment, plants were transferred to a greenhouse (25°C, 16/8 h day/night). NF denotes no flowering observed. Average number of days to flowering indicated the number of days passed before flowering following a vernalization treatment.

Fig. 4 Expression profiles of BdVRN1 (A), BdVRN2 (B) and BdVRN3 (C) genes in the wild type (Bd21) and BdVRN1 RNAi mutant. The relative expression levels of each gene were determined by Real-Time RT-PCR using leaf tissues. Before vernalization, plants were grown at a greenhouse (25°C, 16/8 h day/night). Four-week old seedlings then were transferred to a growth chamber at 4°C (8/16 h day/night) for 0 or 6 weeks. Leaves were sampled before and after vernalization treatment for analysis (BdGAPDH as a loading control).

Fig. 5 Freezing tolerance assessment. A. Freezing tolerance of non-vernalyzed or vernalized plants of Bd21, Bd29-1, and BdVRN1 mutant RNAi-4 was assessed without cold acclimation. Freezing tolerance was assayed by subjecting plants to -5°C and 8/16 h
(day/night) for 12 hours in a growth chamber. Plants were then placed back into a greenhouse (25°C, 16/8 h day/night) and were examined for recovery rate a week later. The non-vernalized plants were 5-week old. Plants used for vernalization treatment were 4-week old before being treated with 6 weeks of vernalization (4°C, 8/16 h day/night). Vernalized plants were then moved to a greenhouse (25°C, 16/8 h day/night). B. Freezing tolerance of non-vernalized or vernalized plants from Bd21, Bd29-1, and RNAi-4 mutant was assessed following cold acclimation. For cold acclimation treatment, plants were grown the same way as for vernalization treatment, except that plants were cold acclimated at a cold chamber at 4°C for 10 days before assessment of freezing tolerance. C. Semiquantitative RT-PCR of BdVRN1 gene in leaves from vernalized or non-vernalized plants of Bd21, Bd29-1, and RNAi-4 mutant. Leave tissues were collected before assessing freezing tolerance for each accession.

Fig. 6 BdVRN1 regulates cold response genes. A. Real-Time RT-PCR analysis of the expression of cold-responsive genes with or without cold treatment. Leaves were harvested 24 hours after a 4 °C cold treatment. B. Real-Time RT-PCR analysis of the cold-responsive genes in the wild type and RNAi mutant after a 6-week vernalization treatment. The expression level of each gene was normalized to that of BdGAPDH gene, and the expression level for each gene in the WT without cold treatment was set to 1.0.
Fig. 1
Fig. 2
Fig. 3

Fig. 4
Fig. 5
Fig. 6
CHAPTER 3 CONSTRUCTION OF A HIGH THROUGHPUT COLD-RESPONSIVE SPECIFIC RNAI LIBRARY FOR THE MODEL GRASS *BRACHYPODIUM DISTACHYON*

A paper to be submitted to *The Plant Journal*

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**Summary**

In plants, the most effective RNA interference (RNAi) strategy is to use long hairpin RNA ( lhRNA) constructs containing inverted repeats (IRs), which silence target
genes based on sequence homology. By now few large-scale tissue-or stress-specific gene silencing by lhRNA constructs has been reported. Here, we developed a novel restriction enzyme-mediated method, Phi29-Amplified RNAi Construct (PARC), which can directly convert a cDNA library into a lhRNA expression library by only three steps (one ligation, one amplification, and one digestion). We used a subtractive library, in which cold-responsive genes are enriched, to construct a PARC library. Random sequencing results showed that the chosen lhRNA constructs could target different genes for silencing. In addition, seventeen lhRNA constructs from the cold-stress RNAi library were delivered together into Brachypodium by Agrobacterium-mediated transformation. Our results indicate that PARC provides a high throughput, rapid and efficient platform for RNAi library construction.

Keywords: high throughput, cold, SSH library, RNAi library, Brachypodium, inverted repeat, hairpin RNA

Introduction

RNA interference (RNAi) is a powerful tool to determine gene function through the generation of loss-of-function mutants. RNAi is a post-transcriptional process triggered by the introduction of a double-stranded RNA (dsRNA), which leads to the specific degradation of mRNA containing the same sequence. In plants, the most effective gene silencing strategy is to use hairpin RNA (hpRNA) constructs containing inverted repeats (IRs), which generate siRNAs (small interfering RNA) that silence target genes based on sequence homology (Waterhouse et al., 1998, Waterhouse and
Helliwell, 2003). Intron-containing hairpin RNA (ihpRNA) construct vector, pHANNIBAL, with intron as a spacer showed a high post-transcriptional gene silencing (PTGS) efficiency in plant, in terms of the proportion of independent transgenic plants showing silencing, and degree of the silencing (Smith et al., 2000, Wesley et al., 2001). pHANNIBAL was a traditional ligase-based vectors, requiring several rounds of enzyme digestions and ligations. Meanwhile, another ihpRNA vector, pHELLSGATE, was developed based on Gateway technology. It requires two rounds of cloning to obtain the final ihpRNA constructs (Wesley et al. 2001). First, PCR products of the gene of interest were cloned into Gateway donor vectors to forming an intermediate vector, thereby making the PCR fragment flanked by the recombination site attB1 and attB2. Second, PCR products of the gene of interest were cloned into Gateway destination vectors by Gateway LR reaction. Based on similar strategy other Gateway-based ihpRNA vectors were developed, such as pIPK and pANDA (Himmelbach et al., 2007, Miki and Shimamoto, 2004). Besides, several hpRNA constructs are developed on the basis of PCR method, such as DA-ihpRNA, OE-PCR, pRNAi-LIC and pRNAi-GG (Xiao et al., 2006, Xu et al., 2010, Yan et al., 2009, Yan et al., 2012). These PCR-based methods are rapid and cost-effective, but are more suitable for silencing single gene rather than a population of unknown genes.

With the development of affordable sequencing technology, the function of tens of thousands of genes could be determined by RNA interference. To generate a plant mutant population with the majority of the genes in the genome silenced, it would be desirable to construct many distinct RNAi constructs in a high throughput manner. To
construct a hairpin RNAi library, short hairpin RNA (shRNA) constructs were
developed by several restriction enzyme-based methods to silence animal target genes,
for example, restriction enzyme-generated small interference RNA (REGS) (Sen et al., 2004), short interference RNA production by enzymatic engineering of DNA (SPEED)
(Luo et al., 2004), and enzymatic production of an RNAi library (EPRIL) system
(Shirane et al., 2004). All these methods involve the ligation of gene of interest to a
hairpin-loop adaptor containing a MmeI. The MmeI-digested fragments are then ligated
to a linear DNA adaptor, allowing strand extension by Bst DNA polymerase (in SPEED)
or primer-extension (in EPRIL) polymerase reaction to synthesize double-stranded DNA,
whereas in REGS, the MmeI-digested fragments were ligated with two hairpin loops and
then were made into double-stranded linear DNA by Phi29 DNA polymerase rolling-
circle amplification (RCA).

Unlike in animals, longer hpRNA (lhRNA) constructs appear to induce more
effective gene silencing than shorter hpRNAs in plants (Helliwell and Waterhouse, 2005,
McGinnis et al., 2005). Based on REGS, a method called rolling circle amplification
mediated hairpin RNA (RMHR) library construction system was reported (Wang et al.,
2008). They designed two different hairpin-loop adaptors ligated with the gene of
interest to amplify by Phi29 DNA polymerase, forming longer hpRNA (lhRNA)
constructs. However, this method requires two cloning steps which could result in low
cloning efficiency. In addition, the short spacer (17 bp) between the IR DNA does not
provide sufficient stability of the expression vector in the bacterial (Wang et al., 2008).
So the method for generating lhRNA construct still needs to be improved.
In recent years, a quite number of genome-wide RNAi libraries have been generated in *Caenorhabditis* elegans (Kamath and Ahringer 2003, Sonnichsen et al. 2005) or *Drosophila* (Boutros et al., 2004, Fisher et al., 2012), but seldom in plants (McGinnis et al., 2007). In barley, a RNAi library was constructed to silence the genes in epidermal cell by Gateway cloning system that is based on biolistic bombardment delivery (Douchkov et al., 2005). It is a high throughput and transient-induced gene silencing (TIGS) method for targeting tissue- or cell-specific genes, where we developed a novel method to generate a high-throughput lhRNA expression library from a population of double stranded cDNAs by a Phi29 polymerase amplification mediated method, referred to Phi29-Amplified RNAi Construct (PARC). This method allows the conversion of a cDNA library, especially a tissue- or stress- specific cDNA library, into an lhRNA expression library by one single ligation and an additional cloning step.

We applied PARC method to *Brachypodium distachyon*, which is a recently developed model for cereals and temperate perennial grasses and its genome information is available now (Draper et al., 2001, Vogel et al., 2010). Determination of the function for each gene in the *Brachypodium* genome is needed for utilization of this species as a model. Although rice is an excellent model for grass species, it is a subtropical species that does not have a well-defined cold acclimation process, a phenomenon by which plants acquire freezing tolerance by a period of exposure to non-freezing low temperatures whereas *Brachypodium* has a well-defined cold acclimation mechanism and is relatively cold hardy (our unpublished data). Cell- or tissue- specific, or stress- specific cDNA populations would allow mutagenesis of a subset of genes preferentially
over other genes in the genome; thereby increasing the chance of a gene of interest being silenced and subsequently isolated. In this study, we generated a suppression subtractive hybridization (SSH) cDNA library from cold treated and non-cold treated *Brachypodium* to focus on transcripts differentially expressed in response to cold. This library was then converted into an lhRNA expression library for gene silencing by PARC. Our results suggested that this novel method provides a high-throughput and reliable platform for making lhRNA interference constructs to determine gene function in large scale.

**Results**

**Production of long hairpin cDNA using PARC**

The main concept of PARC method is to use prepared stem-loop to ligate with cDNA fragments, and the long hairpin cDNA molecules are finally obtained after Phi 29 polymerase amplification and specific enzyme digestion (Figure 1). The stem-loop is obtained from the phagemid pSR252 (Figure 1a). PARC method involves the following 3 steps (Figure 1b): (1) ligation of the stem-loop with cDNA molecules to form closed circular single stranded DNAs (ssDNAs); (2) conversion of the circular ssDNAs into linear double stranded DNAs (dsDNAs) by Phi29 polymerase rolling circle amplification; (3) generation of inverted repeats (IRs) of a cDNA population following digestion by a unique enzyme. IRs are subsequently cloned into a binary expression vector.

The SL was generated from the phagemid, pSR252, containing an inverted repeat structure with multiple cloning sites (MCS). Single-stranded DNA of the pSR252 was
obtained by using the VCSM13 helper phage. The resulting single-stranded DNA, which forms an unsymmetrical dumbbell molecule, was digested by the restriction enzyme, \( PspOMI \), which cuts in the MCS, forming a mini stem-loop structure with 4-nt ‘GGCC’ 5’ overhangs (Figure 1a). Meanwhile, the cDNA population from the SSH library was digested with a \( PspOMI \) compatible enzyme, \( EagI \), and the resulting fragments were ligated with the SL structure to form a dumbbell DNA molecule. A unique restriction site for the enzyme \( XmaI \) was added to the 5’ of the target cDNA during SSH library construction. Ligation products of the SL and cDNA inserts formed single-stranded closed circular dumbbell molecules (Figure 1b, Step 1). The dumbbell DNA molecules were amplified by Phi29 DNA polymerase in a rolling circle DNA replication manner. The resulting high molecular-weight dsDNA molecules were concatemers of the IRs of cDNA inserts and the double-stranded SL (Figure 1b, Step 2). To create lhRNA constructs, the amplification products were digested with \( XmaI \). IRs were purified and inserted into a binary vector to form the final lhRNA expression vector (Figure 1b, Step 3).

**Construction of an SSH cDNA library enriched for cold-responsive genes**

To construct an SSH cDNA library, enriched for cold-responsive genes, the cold treated cDNA population was chosen as the ‘Tester’ and the non-cold treated cDNA population as the ‘Driver’. Differentially expressed genes were enriched by two rounds subtractive hybridizations using excess Driver cDNA population comparing with the Tester population. The differentially expressed cDNAs were exponentially amplified by
two rounds of suppression PCR to create the final SSH library (Figure 2a, b). The second PCR was performed by nested PCR primer. Nested PCR primer 1 included an *Eagl/NotI* recognition site and an *Xmal/SrfI* site, while the nested PCR primer 2 had an *Eagl/EaeI* recognition site. These enzyme recognition sites can be used in subsequent steps during RNAi library construction (Figure 2c).

Subtraction efficiency directly determines the success of the library construction. In our SSH library, only after the abundant constitutive expressed genes were removed, the cold-responsive genes could be enriched. *CBF3* (C-repeat/dehydration-responsive element binding factor 3) is a highly cold inducible gene (Novillo *et al.*, 2012). *CBF3* gene was amplified from the subtracted and unsubtracted cDNAs. The product of the subtracted samples after 20 cycles was as bright as that unsubtracted one after 30 cycles (Figure 2d), indicating that the abundance of *CBF3* cDNA was dramatically increased after subtraction. The SSH cDNA library was cloned into the pGEM-T Easy vector due to the high efficiency in ligation. The ligation mixture was transformed into *E. coli* to make a library with ~10^5 clones. Eleven clones were randomly selected and analyzed by restriction digestion (Figure 2e). *Eagl* digestion resulted in fragments with expected sizes range from 100 to 600bp. Sequencing of the fragments showed that they are all derived from *Brachypodium* genes.

**Conversion of an SSH cDNA library into a RNAi library by PARC**

The SSH cDNA library contained an *Eagl* site on the two adaptors flanking each enriched gene, so after *Eagl* digestion each DNA fragment can generate the symmetrical
overhangs ‘GGCC’ (Figure 2c) which are compatible with the end of PspOMI-digested ssDNA. The SSH library was digested with EagI, and DNA fragments of 200-1000bp in size were isolated, and ligated with the SL to form the closed circle DNA (Figure 3a). The ligation products were linear amplified by Phi29 DNA polymerase, and then fragmented by XmaI, which is the unique cutting site on one adaptor flanking the subtracted DNA population to form the long hairpin population as shown in Figure 1. DNA fragments of 1000-3000 bp in size were separated (Figure 3b) and inserted into the binary expression vector pTF101.1-Ubi, yielding a lhRNA library containing 10^4 clones. Fifty percent of the clones contained the inserts, and thirteen clones were randomly selected and analyzed. As expected, the fragments of 1200-3000bp in size were shown after XmaI digestion (Figure 3c). To confirm the diversity and structure of each lhRNA construct, twenty clones were sequenced (Table 1). The sequencing results indicated that all of twenty clones had the inverted repeat structure, in which the range of the length was 932-1378 bp. BLAST searches were carried on NCBI database using the entry of the sequenced DNA fragments of the insertions, indicating all of the DNA fragments were from Brachypodium (Table 1). Only two of the twenty sequences had the same putative function, but the target genes were different. This indicated that these twenty lhRNA constructs could target twenty different genes for silencing. The results suggest that we could construct an RNAi library with high diversity by PARC.
Discussion

In this study, we developed a new method, PARC, to convert a DNA fragment into an inverted repeat structure based on the enzyme-mediated method. We showed that this method can be applied to construction of an lhRNA expression library from a cDNA library. We also confirmed that the lhRNAi expression vector constructed by this method had strong RNAi effects in *Brachypodium*.

A number of lhRNA constructions methods have been developed for RNA interference in plants, but most of them are based on silencing specific target gene. Wang et.al (2008) reported a rolling-circle amplification-mediated hpRNA (RMHR) construction system can be used for generating libraries of lhRNA constructs from any pool of genes. To confirm the RMHR system, they generated lhRNA constructs targeting the *GUS* gene in tobacco. In their system, two different synthetic hairpin-loop DNA oligos (miniHP1 and miniHP2) were ligated to double-stranded DNA to form a closed circular DNA, which was then amplified by the Phi29 DNA polymerase. One of the loops, miniHP2, serves as spacer in the final lpRNA constructs, but this short spacer (17bp) resulted in relative short DNA fragments (Wang *et al.*, 2008). In our system, the spacer of the final double-stranded lhRNA is the stem-loop (848 bp) generated from the pSR252 directly. The long spacer is suitable for ligation with longer target genes to stable the final lhRNA constructs (Wang *et al.*, 2008). PARC system directly uses the restriction enzyme sites of the SSH cDNA library for ligation with the SL. The SL contains the MCS, giving a lot of options to users to choose the compatible enzyme site for using to ligate with a cDNA library or a single gene. Or the enzyme sites can be
generated by introducing the DNA population into the pGEM-T Easy Vector, which has the recognition site for NotI flanking the insertion site, such as the BRI1 lhRNA construct. We used the method to successfully knock down Brachypodium BRI1 gene, which are required for brassinosteroid signaling (our unpublished data).

Recently, another new method was developed to enzymatically generate a lhRNA expression library from a cDNA plasmid library using a nicking endonuclease, BcaBEST DNA polymerase, and Cre recombinase (Tomimoto et al., 2012). They succeeded in generating an lhRNA library in a silkworm cell line, with about 30-35% of the clones containing IR DNA. With our method, we were able to improve the efficiency with ~50% of the clones were positive and had different inserts with the desired IR structure.

A key advantage of PARC is the high throughput ability to convert an existing cDNA library directly into a lhRNA expression library. Understanding the mechanism of cold acclimation is critically important for improving freezing tolerance in crops including important field crops such as wheat, maize and biomass crops. So we constructed a cold-responsive SSH cDNA library as the source materials for silencing the cold up-regulated genes. In our work, the cold induced gene, CBF3, was highly enriched in the SSH library, indicating the high quality of this cDNA library. In the final RNAi library, eighteen of the twenty lhRNA constructs had the different putative function, and some of them were cold related genes according to the previous research, such as the gene involving the Chlorophyll a-b binding protein (Martino-Catt and Ort, 1992, Wei et al., 2005).
The cold related SSH cDNA library was constructed for the first time in *Brachypodium*, and the cold induced genes were enriched in this library. In our study, the SSH cDNA library was directly converted into an RNA interference library which can silence the known and unknown cold induced genes to generate the mutants. This RNAi library will be a helpful tool for understanding the mechanism of cold acclimation and how plants develop freezing tolerance in *Brachypodium*. Moreover, the PARC system can be applied on other subtracted libraries such as salt or drought related. Finally, as a time and labor saving tool, PARC system can be readily applied on a wide range of crops, especially in which the genomic information is not readily available.

**Experimental Procedures**

**Growth condition and cold treatment**

*Brachypodium*, ecotype Bd21 was used for cDNA library construction. Ten seeds were sown in each 6-inche pot containing Sun Gro Hort soil mix (Bellevue, WA). Plants were grown in a growth chamber at 23°C, 16/8h (day/night) with an irradiance of 400 ± 30μmol m^{-2} s^{-1}. For cold treatment, 3-week-old seedlings were moved into a growth chamber at 4°C for 4 hours, and leaf tissues were then collected and frozen in liquid nitrogen immediately for RNA extraction or stored at −80°C for long-term storage.

**Generation of stem-loop (SL) structure from phagemid vector of pSR252**

pSR252 is a phagemid vector, containing a mini inverted repeat (IR) structure with several restriction enzyme cutting sites (*PspOMI*, *XhoI*, *PstI*, *HindIII* et al.) at its
ends. Single-stranded DNA of the pSR252 was obtained with the help of the VCSM13 helper phage. Briefly, a single colony of XL1-blue containing the pSR252 phagemid was cultured in a 5ml LB liquid medium with Ampicillin and Tetracycline at 37°C overnight. Then the 5ml culture was transferred into a flask containing 200ml LB medium with Ampicillin, and 20µl of the VCSM13 helper phage (final concentration of $1 \times 10^7$ pfu ml$^{-1}$) and cultured at 37°C overnight. The culture was centrifuged at 4 °C, 10,000 rpm for 20 min, and the supernatant was transferred to a new bottle. A three tenth volume of 20% PEG8000/2.5M NaCl was added to the new bottle to precipitate the DNA at 4 °C overnight. Following the precipitation it was centrifuged at 4 °C, at 10,000 rpm for 15 min to yield DNA pellets which were re-suspended in a 5 ml of 0.3 M NaOAc (pH 6.0) and 1mM EDTA. The resulting mixture was extracted by an equal volume of phenol-chloroform for 5 min and centrifuged at 14,000 rpm for 10 min to separate the phases. The aqueous phase was collected and precipitated by 95% ethanol at -20°C for 20 min or longer, followed by a centrifugation at 14,000rpm for 5min. The resulting pellet was washed by 100% and then 70% ethanol sequentially and finally dissolved in a 50μl TE buffer. The ssDNAs were digested with the restriction enzyme PspOMI and digested products were separated in a 1% agarose gel. The ssDNA fragment with a size of about 500bp was excised and purified.
Construction of a cold-responsive suppression subtractive hybridization (SSH)
cDNA library in *Brachypodium*

Total RNA was extracted using TRIzol® Reagent (Life Technologies, Grand Island, NY) from seedling tissues and further purified by NucleoSpin® RNA kit (Clontech, Mountain View, CA) according to the manufacturer’s protocols. mRNA was isolated by NucleoTrap® mRNA Mini Kit (Clontech, Mountain View, CA) following the manufacturer’s instructions.

cDNA suppression subtractive hybridization was performed using the PCR-select cDNA Subtraction Kit (Clontech, Mountain View, CA) following the manufacturer’s instructions. cDNA was synthesized from 2μg of poly A+ RNA generated from non-cold or cold treated seedling tissues. The cDNA obtained from cold-treated seedling tissues was chosen as the tester and the cDNA from non-cold as the driver. The tester and driver cDNA were both digested with *RsaI*. The tester cDNA was then subdivided into two equal portions, and each was ligated with a different cDNA adaptor. The first of the two successive hybridizations is performed in order to equalize and enrich differentially expressed sequences. The two primary hybridization samples are mixed together without denaturing in order to generate PCR templates from differentially expressed transcripts as a result of cold treatment. After two hybridizations, the cold up-regulated sequences were enriched. The entire cDNA population was then subjected to PCR twice to amplify the desired differentially expressed sequences. A second PCR amplification was performed using nested primers to further reduce any background PCR products and
enrich for differentially expressed sequences. Subtraction efficiency was analyzed by PCR.

The subtracted cDNA library was digested by the restriction enzyme *EagI* in order to create a sticky end compatible with *PspOMI* for ligation with the stem-loop structure. The digested products were separated in a 1% agarose gel, and cDNA fragments 2μg of 200-1,000bp were purified for RNAi library construction.

**Ligation of the stem-loop with the subtracted cDNA population and Amplification by Phi29 DNA polymerase**

A total of 2μg subtracted cDNAs were ligated with the stem-loop (molar ratio of stem-loop: cDNA=4:1) by using 1μl of T4 DNA ligase (New England Biolabs, Ipswich, MA) to generate closed circular DNAs. After an overnight incubation at 16°C, the ligation product was precipitated with one tenth volume of 3M NaOAc and 2.5 volumes of 100% ethanol for 30min. Following the precipitation it was centrifuged at 4 °C, at 10,000 rpm for 15 min, and the resulting pellet was dissolved in 89μl ddH2O. To remove the self-ligated stem-loop, 10 units of the restriction enzyme *PspOMI*, and 10μl of the 10x NEBuffer 4 (New England Biolabs, Ipswich, MA) were added to 89μl ligation products and incubated at 37°C for 2 hours. The digestion products were precipitated as described above. The precipitated product was centrifuged at 4 °C, at 10,000 rpm for 15 min and the pellets were dissolved in 30μl TE buffer.

The ligation product was amplified by using the IllustraTM TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences, Pittsburgh, PA) following the
manufacturer's instructions. Briefly, 10µl of the ligation product was mixed with 100µl of the Phi29 reaction buffer, 100µl of the sample buffer and 2µl of the Phi29 DNA polymerase. After an overnight incubation at 30°C, the amplified product was precipitated with one tenth volume of 3M NaOAc and 2.5 volumes of 100% ethanol for 1hr. The precipitated product was centrifuged at 4 °C, at 10,000 rpm for 15 min and the resulting pellet was dissolved in 120 µl ddH2O.

Construction of a long hairpin RNA interference library for Brachypodium

After Phi29 amplification, the double stranded DNAs were digested with the restriction enzyme XmaI. The inverted repeats of cDNA inserts ranging from 1000-3000 bp were separated in a 1% agarose gel and purified. The purified IRs were ligated with the binary vector pTF101-Ubi for plant transformation. pTF101-Ubi was derived from base vector pTF101.1 MCS version (Paz et al., 2004) by replacing the CaMV 35S promoter with the maize ubiquitin promoter. The binary vector was introduced into the Agrobacterium tumefaciens strain C58C1 by electroporation.

Genetic transformation of Brachypodium with the RNAi library

The RNAi constructs were introduced into the B. distachyon by Agrobacterium-mediated transformation (Vogel and Hill, 2008). The selection medium contained Timentin (bioWORLD, Dublin, OH) at 150 mg/L to suppress Agrobacterium growth and selective agent glufosinate (Chem Service, Inc. West Chester, PA) at 4 mg L⁻¹ for two cycles under dark at 23°C, each of which lasted two weeks to kill untransformed calli.
Resistant calli were moved into a regeneration medium containing KT at 1 mg L$^{-1}$ and glufosinate at 4 mg L$^{-1}$ under 24h light with an irradiance of 180 ± 30μmol m$^{-2}$ s$^{-1}$. The shoots started to appear after 7-10 days. Rooting took place on the MS medium supplemented with 0.1 mg L$^{-1}$ NAA and 4 mg L$^{-1}$ glufosinate. When plantlets are large enough, carefully move rooted plantlets into 6-inch pots containing Sun Gro Hort soil mix (Bellevue, WA). Plants were grown in a growth chamber at 23°C, 16/8h (day/night) with an irradiance of 480 ± 30μmol m$^{-2}$ s$^{-1}$.

Transgenic plants were confirmed by PCR using primers designed from the sequence of a bar (phosphinothricin acetyl transferase) gene (forward 5’GGATCTACCATGAGCCCAGA 3’, reverse 5’ GAAGTCCAGCTGCCAGAAAC 3’) present in the binary vector. DNA fragment of each inverted repeat was sequenced. Forward primer was designed from upstream region of the inserts (forward 5’TGTAGTCCATGGTGAAA 3’), and reverse primer was designed from the stem-loop region (reverse 5’ CATGACGACCAAGCCATGTA 3’).

Acknowledgements

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References


### Tables

Table 1. The sequencing results of the hairpin RNA library

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<td>Bradi3g34400</td>
<td>120</td>
<td>100</td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>20</td>
<td>Bradi4g28137</td>
<td>117</td>
<td>100</td>
<td>Uncharacterized</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1. Schematic diagram of how the lhRNA construct is generated by PARC.**
(a) Double stranded plasmid DNA pSR252 contains a mini inverted repeat (IR) structure with multiple cloning sites (MCS) at its ends. Single-stranded plasmid DNA can be obtained with the use of the VCSM13 helper phage. The stem-loop structure is recovered by digestion with a restriction enzyme in the MCS, for example *PspOMI*. (b) Step 1, a cDNA population enriched for genes expressed in response to a particular stress such as cold is obtained by a digestion with a *PspOMI* compatible enzyme, for example *EagI*. Digested fragments are then ligated with the stem loop to form dumbbell DNA molecules. A Unique restriction enzyme site, for example *XmaI*, is added at either the 5’ or 3’ end of all cDNA molecules. Step 2, dumbbell DNA molecules are amplified by the Phi 29 DNA polymerase in a rolling circle DNA replication manner, which result in high molecular-weight dsDNA molecules (up to 40kb), concatemers of the IRs of cDNA inserts and the double-stranded stem-loop. Step 3, digestion of the amplified DNA with *XmaI* releases the IRs of the target genes and stem-loops. The former can be separated on a gel, recovered and directly cloned into a binary expression vector.

**Figure 2 SSH cDNA library construction.** (a) First round PCR amplification was performed after hybridization. PCR products use PCR primer1 for PCR amplification. After first amplification there is no great difference between the unsubtracted and subtracted population. (b) Second round PCR amplification using nested PCR primers. After second amplification, the subtracted genes are greatly enriched. (c) Diagram
depicting the cDNA population digested with EagI enzyme, generating sticky ends to ligate with stem-loop. (d) Increase of CBF3 abundance by PCR-select subtraction. PCR was performed using the second PCR product of unsubtracted (lane 1-4) or subtracted (lane 5-8) by CBF3 primers. Lane 1 and 5: 15 cycles; lane 2 and 6: 20 cycles; lane 3 and 7: 25 cycles; lane 4 and 8: 30 cycles. The CBF3 increase significantly after subtraction. (e) Gene fragments of the SSH cDNA library were digested with EagI. The majority of the inserts in the SSH cDNA library has a size ranging from 100-600bp.

**Figure 3. Generation of RNAi library.** (a) Lane 1, SSH cDNA library was digested with EagI and DNA fragments of 200-1000bp were gel-purified; lane M, 1kb ladder; lane 2, ssDNA was digested with PspOMI and SL was separated. (b) Lane M, 1kb ladder; lane1, the ligation products of SSH cDNA library with SL were amplified by Phi29 polymerase, and further were digested with Xmal. Digested fragments of 1000-3000bp were purified from gel and cloned into binary vector forming the final hairpin RNA library; lane 2, SL self-ligation products were amplified by Phi29 polymerase, and digested with PspOMI, forming more double strands SL. (c) Thirteen clones were selected and digested with Xmal generating 1200-3000bp fragments.
Figure 1
Figure 2

(a) 

(b) 

(c) 

Figure 3
CHAPTER 4  Down-regulation of BdBR11, a BR11 homolog from Brachypodium distachyon, enhances drought tolerance

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Summary

Brassinosteroids (BRs) play important roles in plant growth, development, and responses to a range of environmental cues. BR biosynthesis and signaling have been the subjects of intensive studies in Arabidopsis. Although the molecular basis of the
regulatory roles that BRs play during plant growth and development is well understood, the effect of BR signaling on plant stress tolerance especially in response to drought stress is not well documented and remains poorly understood.

We isolated a BRI1 (BRASSINOSTEROID INSENSITIVE 1) homolog gene, BdBRI1 from Brachypodium distachyon, a model for temperate grasses and created and characterized RNA interference (RNAi) knockdown mutants for BdBRI1 in Brachypodium.

The loss-of-function BdBRI1-RNAi mutants exhibited phenotypes typical of BR insensitive mutants including dwarfism, short internode, narrow, and short leaf, and reduced expression of BR signaling genes, BES1, BZR1, BLE2, and enhanced expression of BR biosynthesis genes D2, CPD and DWF4. Moreover, BdBRI1 RNAi mutants exhibited enhanced drought tolerance with constitutive expression of drought-responsive genes, ERD1 and RD26, two putative targets of the transcription factors BES1 and BZR1.

Our results suggest that BR signaling and biosynthesis are conserved among Arabidopsis, rice, barley and Brachypodium, and that BR signaling components play an important role in plant drought tolerance by regulating expression of the key drought-responsive genes.

**Key words:** brassinosteroid, signaling, BRI1, Brachypodium distachyon, drought tolerance, knockdown
Introduction

Brassinosteroids (BRs) play diverse roles in plant growth, development, and responses to different environmental factors. Brassinolide (BL) is the first BRs isolated from pollen of *Brassica napus* (Grove *et al.*, 1979), and BRs have been recognized as the sixth class of plant hormones because of its potent biological activity. Early works found that application of exogenous BL affects cell expansion, cell division, vascular differentiation, male sterility, senescence, and modulation of stress responses (Mandava, 1988; Clouse & Sasse, 1998; Divi & Krishna, 2009). Meanwhile, BR-deficient (loss of function in BR biosynthesis) or BR insensitive mutants (loss of function in BR perception) were identified and found to display similar phenotype of dwarfism with compact stature, small and dark green leaves, delayed flowering and senescence, and decreased male fertility (Clouse *et al.*, 1996; Li *et al.*, 1996; Szekeres *et al.*, 1996). BR biosynthesis and BR signaling have been the subjects of extensively studies in *Arabidopsis* and rice (*Oryza sativa*), and significant progresses have been made (Li, 2010; Clouse, 2011; Zhu *et al.*, 2013).

BRI1 (BRASSINOSTEROID INSENSITIVE 1) is a transmembrane serine/threonine kinase receptor with an extracellular domain containing leucine-rich repeats (LRRs) and an island domain that binds BR (Clouse *et al.*, 1996; Li & Chory, 1997; He *et al.*, 2000; Wang *et al.*, 2001; Kinoshita *et al.*, 2005; Hothorn *et al.*, 2011; She *et al.*, 2011). In the absence of BRs, BKI1 (BRI1 KINASE INHIBITOR 1) binds to the intracellular domain of the BRI1 to keep it from associating with co-receptor BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE1) (Li *et al.*, 2002; Nam & Li, 2002; Wang
In contrast, bindings of BRs to the extracellular domain of BRI1 promote dissociation of BRI1 from BKI1 and result in its association with BAK1, thereby triggering a signal transduction cascade (Nam & Li, 2002; Wang et al., 2005; Tang et al., 2008; Jaillais et al., 2011; Wang et al., 2011; Gou et al., 2012). Activated BRI1 phosphorylates BSK1 (BR SIGNALING KINASE 1) and CDG1, which subsequently activates BSU1 (BRI1-SUPPRESSOR 1) to dephosphorylate and repress BIN2 (BRASSINOSTEROID INSENSITIVE 2), a negative regulator in the BR signaling pathway (Li & Nam, 2002; Mora-Garcia et al., 2004; Tang et al., 2008; Kim et al., 2009; Yan et al., 2009; Kim et al., 2011). The inhibition of BIN2 leads to the accumulation of the two transcription factors BES1 (BRI1-EMS SUPPRESSOR 1) (also called BZR2) and BZR1 (BRASSINAZOLE RESISTANT 1) from suppression to regulate down-stream target genes (Li & Nam, 2002; Wang et al., 2002; Yin et al., 2002; Zhao et al., 2002). BR biosynthesis is regulated by a feedback mechanism. When BRs reach to a level sufficient to maintain plant growth and development, several BR biosynthesis genes are repressed by BES1 and BZR1 to down regulate the BR biosynthesis genes (Ye et al., 2011).

Recently, studies using ChIP –chip (Chromatin immunoprecipitation followed by genomic tilling arrays) have identified many BES1 and BZR1 target genes, which are involved in plant growth, other hormone-mediated signaling, responses to light, abiotic or biotic stresses (Sun et al., 2010; Yu et al., 2011). Meantime, it is reported that manipulation of the BR signaling genes can improve plant stress tolerances (Koh et al., 2007; Divi & Krishna, 2009; Kim et al., 2010; Yang et al., 2010). The microarray
analysis of bri1-9 mutant in Arabidopsis revealed that the increased cold tolerance in bri1-9 may because of the constitutively upregulated expression of some cold-inducible genes (Kim et al., 2010). However, the mechanism of BR signaling on stress tolerance and how BR signaling components regulate the induction of the stress-responsive genes remains to be addressed.

*Brachypodium distachyon* has recently been established as a model for cereals and temperate grasses because of its sequenced small genome (~270M), a small stature, short life cycle, self-fertility, and simple growth requirement (Draper et al., 2001; Vogel et al., 2010; Brkljacic et al., 2011). It is also a good system for various stress tolerances study because of its natural variations in these traits (Luo et al., 2011; Li et al., 2012). In this study, we isolated a BRII-like gene, BdBRII from *Brachypodium* and generated the BdBRII-RNAi mutants to knock down the BdBRII expression level. The RNAi mutants showed a typical BR-insensitive phenotype as dwarfism with compact stature, narrow and short leaf, short internode, decreased BR response, and modulated expression level of BR-related genes. Moreover, we found that knockdown of the BdBRII expression conferred highly increased drought tolerance in transgenic plants. The drought-responsive genes (*RD26* and *ERD1*), two putative target genes of transcription factors BES1 and BZR1 were constitutively expressed (Simpson et al., 2003a; Fujita et al., 2004). To our knowledge, this is the first report of BR signaling pathway in *Brachypodium*. Therefore, our results not only first confirmed that the BR genes and signaling are conserved between *Arabidopsis*, rice and *Brachypodium*, but also showed
crosstalk exists between BR signaling pathway and drought stress response in

*Brachypodium*.

**Material and Methods**

**Plant materials and growth condition**

Seeds of *B. distachyon* (Bd21) were kindly provided by John Vogel at USDA-ARS. Seeds were grown in a growth chamber at 23°C, 16/8h (day/night) with an irradiance of 280 ± 30 μmol m$^{-2}$s$^{-1}$. For gene isolation, leaves of the 3-week-old seedlings were collected and frozen in liquid nitrogen immediately for RNA extraction or stored at −80°C for long-term storage.

**Identification of BRI1 gene in *B. distachyon* and phylogenetic analysis**

BLAST search of *B. distachyon* genome was carried on the Phytozome V9.0 server for *Brachypodium distachyon* (http://www.phytozome.com/search.php?show=text&method=Org_Bdistachyon) using the query sequence of *Arabidopsis* BRI1 protein (AEE87069). Multiple sequence alignment was carried out by ClustalW program on GenomeNet server (http://www.genome.jp/tools/clustalw/). Phylogenetic analysis was performed by ClustalW with Neighbor Joining method on the Phylogeny.fr server (http://www.phylogeny.fr) for BRI1 homolog proteins from different species, including *Arabidopsis* (AtBRI1, AAC49810; AtBRL1, Q9ZWC8; AtBRL2, Q9ZPS9; AtBRL3, Q9LJF3), rice (OsBRI1, BAB68053; OsBRL1, BAD34326; OsBRL2, AAK52544;
OsBRL3, BAD01717), barley (HvBRII, BAD06331), tomato (LeBRII, AAN85409; SR160, Q8GUQ5), tobacco (NbBRII, ABO27628), pea (PsBRII, BAC99050), and potato (StBRII, ABO27627).

**RNAi vector construction and generation of RNAi mutants**

To generate a long hairpin RNAi construct, primers were designed from the mRNA sequence of *BdBRII* (Bradi2g48280). The 3’ end coding region of *BdBRII* (837 bp) was amplified using the primers (forward 5’
GATAACCCAGCTCTTTTCTCGACACTC3’ and reverse 5’
TGTGGTGGAGAAATGCCAATCCT3’). An AscI recognition site (GGCGCGCC) was introduced to the 5’ end of forward primer ( 5’GGCGCGCCGATAACCCAGCTCTTTTCTCGACACTC 3’) to generate the inverted repeat structure later. The PCR reaction was performed as the following: 94 °C for 3min, and 35 cycles of 94 °C for 20 sec, 59 °C for 30 sec, 72 °C for 50 sec, and a final extension of 72°C for 1min. The PCR product was separated on a 1% agarose gel. The gene fragment was cloned into the pGEM-T Easy Vector (Promega, Madison, WI) using the T4 DNA ligase (New England Biolabs, Ipswich, MA). The clone was verified by DNA sequencing analysis.

pSR252, a phagemid, was kindly provided by Richard Jorgensen. Its single strands can be rescued by the VCSM13 helper phage. The ssDNA of pSR252 was digested with *PspOMI* to form a mini stem loop structure with a 4- nucleotide 5’ overhang ‘GGCC’. The *BdBRII* fragment was digested with *NotI*, also generating a
5’overhang “GGCC”, which is compatible with the end of PspOMI-digested mini stem loop. The ligation of stem loop with the BdBRI1 fragment can form a closed circular DNA, which was then amplified by the Phi29 polymerase (GE Healthcare Life Sciences, Pittsburgh, PA). The amplified product was digested with AscI to generate the double-stranded inverted repeats (IR) of BdBRI1 gene. The IR fragments were ligated to a binary expression vector pFY32 that had been digested with AscI, generating the pFY32-BRI1 RNAi construct under the control of two copies of the CaMV 35S promoter and the nopaline synthase (nos) terminator. pFY32 was modified from pMDC32 (Curtis & Grossniklaus, 2003) by performing LR recombination reaction between the entry clone pENTR™/D-TOPO® (Life Technologies, Grand Island, NY) and the Gateway destination vector of pMDC32 to eliminate the attR1-ccdB-attR2 cassette and meanwhile adding an AscI site. The construct was verified by sequencing.

The pFY32-BRI1 vector was introduced into Agrobacterium C58C1 strain and transformation of Brachypodium was carried out following the protocol developed by Vogel and Hill (2008). The selection medium contained Timentin (bioWORLD, Dublin, OH) at 150 mg L\(^{-1}\) to suppress Agrobacterium growth and selective agent hygromycin B (bioWORLD, Dublin, OH) at 40 mg L\(^{-1}\) to kill untransformed calli for two cycles under dark at 23°C, each of which lasted two weeks. Resistant calli were moved into a regeneration medium containing MS basal medium KT at 1 mg L\(^{-1}\) and hygromycin at 10 mg L\(^{-1}\) in a tissue culture chamber at 23°C, 24h (light) with an irradiance of 180 ± 30 μmol m\(^{-2}\) s\(^{-1}\). Shoots started to appear after 7-10 days. Rooting took place on the MS medium supplemented with 0.1 mg L\(^{-1}\) NAA and 10 mg L\(^{-1}\) hygromycin. Well-rooted
plantlets were carefully moved into 6-inch pot containing Sun Gro Hort soil mix (Bellevue, WA). Plants were grown in a growth chamber at 23°C, 16/8h (day/night) with an irradiance of 400 ± 30 μmol m⁻² s⁻¹.

Transgenic plants were screened by PCR using primers (HPT-F 5’GAATTCAGCGAGGCTG 3’, HPT-R 5’ ACATTGTGGAGCCGAAA 3’). Thirteen independent T₀ lines were confirmed by PCR analyses. In the independent lines, RNAi-10 and RNAi-27 segregate PCR-positive and PCR-negative in progenies in the ratio 3:1, suggesting the integrations of the transgenes at a single locus in each of the two lines. The homozygous plants of T₃ RNAi-10 and RNAi-27 lines were chosen for further analyses.

**Microscopy**

For light microscopy (LM), stems were fixed in FAA (formalin-acetic acid-alcohol) for several days at 4°C. They were dehydrated in a graded ethanol series, cleared with xylene, infiltrated and embedded using Paraplast paraffin (Fisher Scientific, Pittsburgh, PA). Sections were made using an A/O 820 rotary microtome (Fisher Scientific, Pittsburgh, PA). Sections were cut at 10μm, collected onto slides, deparaffinized and stained with toluidine blue-o, then dehydrated, cleared and coverslipped. Digital images were collected using a Zeiss Axiocam HRC on a Zeiss AxioPlan II (Carl Zeiss Inc, Thornwood, NY) compound microscope.
**Analysis of BL sensitivity**

The lamina inclination assay was performed as described previously in rice (Wada et al., 1981). Seeds were grown on moisturized filter papers for 1 week in a tissue culture chamber at 23°C, 16/8h (day/night) with an irradiance of 180 ± 30μmol m⁻² s⁻¹. One μl of ethanol containing 0, 10, 100, 1000 ng of BL was spotted onto the joint between the leaf blade and sheath of the first leaf (from bottom). Images were captured 24h after BL treatment and the angles of lamina joint were measured by using ImageJ software.

To determine coleoptile elongation response, seeds were germinated on MS basal solid medium supplemented with 0.1 μM of BL. After growing 5 days in a dark growth chamber at 23°C, the lengths of coleoptile were measured. The procedure of coleoptile elongation response was performed as described previously in rice (Duan et al., 2006).

**Drought stress treatment**

For drought treatment, ten seeds of Bd21 and BRI1-RNAi mutants were sown in 8-inch pots containing Sun Gro Hort soil mix (Bellevue, WA) in the greenhouse at 23°C, 16/8h (day/night) with an irradiance of 800 ± 30μmol m⁻² s⁻¹. Three-week-old seedlings were subjected to drought stress by withholding water until the soil moisture content reached within the 14-15% range. Leaf chlorophyll fluorescence was measured by the ratio of Fv/Fm under dark on three randomly selected leaves in each plant using a fluorescent meter (PAM-2000, Heinz Walz GmbH, Germany). Leaf water content (LWC) was determined based on the following formula: LWC= (FW-DW)/FW x100,
where FW is the leaf fresh weight, DW is leaf dry weight. When the soil moisture content was within the 14-15% range, plants were removed 1cm above the soil surface and dried in an oven at 67°C for 5 days. Soil moisture content (SMC) was measured by the following equation: SMC = (SFW - SDW)/SFW x 100, where SFW is the soil fresh weight, SDW is soil dry weight. We measured SFW for every three days with plants grown in the pot until the leaf became severely wilted. When plant materials were removed, the remaining soil was dried for 5 days at 67 °C, and was weighted as SDW. Once soil moisture content reached the 14-15% range, watering was resumed for plant recovery. One week after rewatering, the number of the surviving plants was recorded. All experiments were repeated three times.

**Real-Time RT-PCR analysis**

For BL treatment, 2-week-old seedlings were sprayed with 10 ml of 1 μM BL and leaf tissues were collected 4 hours after treatment. For drought treatment, leaf tissues of the treated seedlings were collected when plants showed leaf curling (SMC, 40-42%). Total RNA was extracted with TRIzol® Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 5 μg total RNA as the template by using the SuperScript® III First-strand Synthesis System (Invitrogen, Carlsbad, CA) and quantitative RT-PCR was carried out on the Eco Real-Time PCR System (Illumina, Inc., San Diego, CA) using the SYBR® GreenER qPCR SuperMix™ kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity of PCR products was determined at the end of each cycle by the Eco Software v4.0 (Illumina,
Inc., San Diego, CA). The expression level of each gene was normalized to that of 
*BdGAPDH* gene, and the expression level for each gene in the wild type without 
treatment was set to 1.0. Primers used for PCR amplification are listed (Table S2). To 
identify the genes involved in BR pathway and drought response in *Brachypodium*, the 
amino acid sequences of *Arabidopsis* genes (*BES1, BZR1, CPD, DWF4, ERD1, 
*DREB2A, RD29B, RD26, RD22, COR47*) and rice genes (*BLE2, D2*) were used to search 
all possible homologs in the *B. distachyon* genome by the BLASTP tool on the 
Phytozome server. The one with the lowest E-value was used to design primers for 
isolation of homologous genes in *Brachypodium* (Supporting Information Table S3).

**Statistical analysis**

Statistical analysis was performed using the JMP package program version 
10.0.0. For Fig. 3d, the data was analyzed by one-way t-test. Error bars represent 
standard error (*, p <0.05). For Fig. 2, 3b-c, and 4-7, the data were analyzed for each 
pair using Student’s t-test. The values are reported as means ± standard error (SE) for all 
results. Differences were considered significant at *P*<0.05. Levels are not connected by 
same letter are significantly different.

**Results**

**Isolation of a *Brachypodium BRII* homolog gene, *BdBRII***

By BLAST search, three BRII-like proteins, Bradi2g48280, Bradi3g21400, and 
Bradi4g27440, were identified in *Brachypodium*. We compared the amino acid
sequences of BRI1 or BRL (BRI1-Like) proteins with those from other species, including *Arabidopsis*, pea, tobacco, rice, tomato, potato, and barley (Fig. S1). The multiple alignment results indicated that they all contain the conserved domains including several leucine-rich repeat (LRR) motifs, a transmembrane domain and a cytoplasmic kinase domain. The numbers of the LRR motifs are 22 in Bradi2g48280, 23 in Bradi4g21440, and 26 in Bradi3g21400, compared with 25 in the *Arabidopsis* BRI1. The predicted Bradi2g48280 protein shares 83% amino acid identity with the OsBRI1 in rice, and 89% with the HvBRI1 in barley, which is consistent with the established phylogenetic relationship (Table S1) (Davidson *et al.*, 2012). While the other two BRI1-like proteins from *Brachypodium* had a low amino acid identity to the BRI1s. Besides, the locations of the missing or added LRR motifs vary in these three BRI1-like proteins. Bradi2g48280 lacks three LRR motifs, each corresponds to the seventh, ninth and tenth LRR of the *Arabidopsis* BRI1, but it is same as the OsBRI1 in rice and HvBRI1 in barley (Fig. 1a).

A phylogenetic tree was constructed based on the result of multiple sequences alignment (Fig. 1b). BRI1s/ BRLs were divided into three groups. Bradi2g48280 was classified into the same group as the *Arabidopsis* BRI1, the OsBRI1 and HvBRI1. Bradi4g27440 belongs to the same group as AtBRL1 and AtBRL3 of *Arabidopsis* and OsBRL1 and OsBRL3 in rice, and Bradi3g21400 belongs to the same group as AtBRL2 and OsBLR2. These results strongly suggested that Bradi2g48280 is the BRI1 homolog and is thereafter referred to BdBRI1.
Characterization of *BdBRI1*-RNAi mutants

To determine the function of the putative BR receptor gene in *Brachypodium*, we generated a *BdBRI1* RNAi construct by the PARC (Phi29-Amplified RNAi Construct) method for producing *BdBRI1*-RNAi lines (Fig. 2a). Thirteen independent T₀ transgenic lines were generated and confirmed by PCR analysis and two of them, RNAi-10 and RNAi-27 exhibited a typical dwarf phenotype of BR loss-of-function mutants. Real-Time RT-PCR showed that *BdBRI1* transcript levels were reduced by 79 ± 0.02 % in RNAi-10 and 88 ± 0.04 % in RNAi-27 (Fig. 2b, c), and the other two *BRI1*-like genes in Brachypodium still kept the similar level relative to the wild type (Fig. S2).

The RNAi-10 and RNAi-27 lines had a compact stature with fewer tillers, shorter internodes, narrow and short leaves (Fig. 3a). The average tiller number was reduced to 5 per seedling in RNAi-10 and 4 per seedling in RNAi-27 mutant from 7 in the wild type (Fig. 3b). The average length of each internode in RNAi-10 mutant was reduced to 0.86 ± 0.03cm, while in wild type approximately 2.24 ± 0.01cm (Fig. 3c). Microscopic observation of the internode longitudinal sections indicated that the cell length was decreased in the RNAi mutant relative to wild type (Fig. 3d, e). The severities of the RNAi-10 and RNAi-27 phenotypes correspond to the *BRI1* expression levels (Fig 2b, c). These results strongly suggested that *BRI1* plays a significant role in *Brachypodium* growth and development.
Reduced sensitivity of *BdBRI1*-RNAi mutants to BL

Further, we tested the sensitivity of RNAi mutants to BL using lamina inclination experiment (Fig. 4a). If a mutant is less sensitive to BRs, the degree of bending between the leaf blade and sheath of the mutant plants should be less than that in the wild-type plants. The results of RNAi-10 mutant were shown here (Fig. 4a, b). As expected, in the wild type, the angle of bending was increased with the increasing concentrations of BL. Under the 10 ng μl⁻¹ of BL treatment, no significant change was observed in wild type. But as the BL concentration increase to 100 ng μl⁻¹, the bending angle was greatly increased from 60 degree at 10 ng μl⁻¹ to almost 90 degree. The angle was farther increased to 100 degree at 1,000 ng μl⁻¹ (Fig. 4a, b). In contrast, the angle of bending of the leaves increased only slightly in RNAi-10 mutant from 28 degree at 10 ng μl⁻¹ to 36 degree at 100 ng μl⁻¹, and to 42 degree at 1,000 ng μl⁻¹. The increase was much less than that of the wild type under the same concentration (Fig. 4a, b). In the wild type, both coleoptiles and root elongation was repressed by BL due to the feedback regulation of BR signaling pathway (Fig. 4c, d, e). In contrast, RNAi mutants did not exhibit the same suppressive effect of BL as observed in the wild type (Fig. 4c, d, e). RNAi-27 showed similar results in above mentioned experiments. Taken together, these results indicated that the BL sensitivity was reduced in the *BRI1* RNAi mutants due to the greatly reduced expression of the *BdBRI1* gene.
Altered expression of BR-related genes in *BdBRII*-RNAi mutants

Using Real-Time RT-PCR, we examined the expression levels of the genes known to be involved in BR signaling *Brachypodium BRI1, BZR1, BES1, BLE2 (BL ENHANCED 2)* and BR biosynthesis *D2 (DE-ETIOLATED 2), CPD (CONSTITUTIVE PHOTOMORPHOGENESIS), DWF4 (DWARF4)* (Table S3). The BR signaling genes were all reduced in the RNAi-10 mutant in comparison with the wild type, whereas all the BR biosynthesis genes had higher expression levels in the RNAi-10 mutant than in the wild type (Fig. 5).

With BL treatment, the signaling genes *BES1, BZR1* and *BLE2* were up regulated greatly, whereas the biosynthesis genes *D2, CPD* and *DWF4* were down regulated in the wild type (Fig. 5). This is consistent with the feedback regulation (positive or negative) observed in *Arabidopsis* and rice. In the RNAi-10 mutant, the expression levels of the signaling genes were increased slightly under BL treatment, but much lower than the wild type. BL reduced the expression levels of all BR biosynthesis genes in RNAi mutant, and their levels were still higher than that in the wild type with BL treatment (Fig. 5). Similar results were observed in the RNAi-27 mutant.

Enhanced drought tolerance in *BdBRII* RNAi mutants

Enhanced drought stress tolerance was observed in RNAi mutants. Because the RNAi mutant seedlings are smaller than the wild type, it took 19 days of withholding water to reach a soil moisture level 14-15% for the mutants, while it only took 12 days to reach a similar level of moisture for wild type. When soil moisture content reached the
14-15% in range, the wild type started to show severe leaf wilting (Fig. 6a). In contrast, the RNAi-10 mutant did not show severe leaf wilting even withholding water for 19 days when soil moisture content reached the same range.

Chlorophyll fluorescence can be used to estimate the efficiency of photosynthesis by measuring the value of Fv/ Fm, which is the ratio of variable fluorescence to maximal fluorescence. To compare drought tolerance between the wild type and RNAi mutants, we measured the Fv/ Fm in both the wild type and RNAi mutant seedlings under the same soil moisture condition (Fig. 6c). The Fv/Fm value showed a dramatic decline in the wild type with decreased soil moisture, while the value in the RNAi-10 mutant had a slower decline. When the soil moisture content reached 14-15%, the Fv/Fm in RNAi-10 mutant was reduced to 0.5 ± 0.09, which was almost 2 fold higher than that in the wild type. Moreover, the leaf water content (LWC) of RNAi-10 seedlings (47.2 ± 2.1%) was much higher than that of the wild type plants (24.2 ± 1.2%) when the soil moisture content was at 14-15% (Fig. 6d). After rewatering, 77.8% of the RNAi-10 mutant plants were recovered 19 days after withholding water, whereas, only 22.5% of the wild type plants were recovered 12 days after withholding water (Fig. 6b, e). These results indicated that knockdown of the transcripts of BdBR11 conferred increased tolerance to drought stress in Brachypodium.

**Increased expression of drought-responsive genes in BdBR11 RNAi mutants**

To determine if any of the drought-responsive genes displays differential expression in the RNAi mutants, we analyzed several reported drought-responsive genes
by real-time RT-PCR. The CBF/DREB (C-repeat binding factors/DEHYDRATION RESPONSIVE ELEMENT BINDING) genes are essential for induction of the expression of various stress-responsive genes in the ABA-independent dehydration or cold stress-tolerance pathways, because the CBF/DREB genes activate the expression of many target genes that are responsible for controlling cell osmoprotection and metabolism (Catala et al., 2003; Dubouzet et al., 2003; Ito et al., 2006; Lata & Prasad, 2011). In the DREB2 subfamily, DREB2A is a transcription factor induced by drought and heat stress in Arabidopsis, rice, soybean and maize (Simpson et al., 2003a; Sakuma et al., 2006a; Sakuma et al., 2006b; Qin et al., 2007; Cui et al., 2011; Mizoi et al., 2013). The RD (RESPONSIVE TO DESICCATION) genes including RD29B, RD22, and RD26, are also induced by drought stress through the regulation of the transcription factor AREBs (ABA-RESPONSIVE ELEMENT-BINDING PROTEINS) or DREBs (Yamaguchi-Shinozaki & Shinozaki, 1994; Nakashima et al., 2006; Msanne et al., 2011). Another drought-responsive gene, ERD1 (EARLY RESPONSIVE TO DEHYDRATION STRESS 1) is involved in the proteolysis in plastids under dehydration conditions (Kiyosue et al., 1993; Zheng et al., 2002). P5CS encodes an enzyme, delta 1-pyrroline-5-carboxylate synthetase, involved in the biosynthesis of proline, which is critical for prevention from dehydration stress damage such as drought or salt by increasing osmotic potential of cells. Previous researches showed the expression of P5CS was highly induced by drought stress (Hu et al., 1992; Yoshiba et al., 1995; Verslues et al., 2006).

With drought treatment, all above mentioned drought-responsive genes were induced in both RNAi-10 mutant plants and the wild type (Fig. 7), but the ERD1, RD26
and $P5CS$ had much higher level in the mutants than that in the wild type. And interestingly, $ERD1$ and $RD26$ were highly expressed in the RNAi-10 mutant as well as in the RNAi-27 even without drought treatment. These results suggested that the inducible expression pattern of the drought-responsive genes were not changed in the RNAi mutants relative to wild type. Moreover, knockdown of the $BdBRI1$ gene resulted in constitutively express of $ERD1$, $RD26$ and $P5CS$.

**Discussion**

$BRI1$ genes have been identified from *Arabidopsis*, rice, tomato, barley, and pea (Yamamuro *et al.*, 2000; Montoya *et al.*, 2002; Chono *et al.*, 2003; Nomura *et al.*, 2003; Nakamura *et al.*, 2006). Three homologous genes of $BRI1$ have been reported in *Arabidopsis*, $BRL1$, $BRL2$, and $BRL3$. $BRL1$ and $BRL3$ are functional BR receptors, but they are restricted to vascular cells (Cano-Delgado *et al.*, 2004). $BRL2$ was identified as Vascular Highway 1(VH1) without brassinolide-binding ability. It is required to maintain provascular differentiation in the leaves (Clay & Nelson, 2002). In rice, while $OsBRI1$ gene is expressed in all organs, the other two $BRI1$ homologs, $OsBLR1$ and $OsBLR3$ are highly expressed only in the roots (Nakamura *et al.*, 2006). Based on the phylogenetic tree, we speculated that the other two $BRI1$-like genes in *Brachypodium*, Bradi4g27440 and Bradi3g21400, are the homolog of $BRL1/BRL3$ and $BRL2$ respectively, but this remain to be confirmed.

Sequence alignment of $BRI1$ homologs showed that $BRI1$ homologs from the monocot species rice, wheat, and *Brachypodium*, are all missing three LRR motifs.
relative to the dicot species *Arabidopsis*, pea, tomato, potato and tobacco (Fig. 1a). It may result from the evolutionary diversity of BRI1 between dicots and monocots. Apart from the structure, the function of *BdBRI1* was also conserved in *Brachypodium*. *BdBRI1* knockdown mutants exhibited the typical dwarfism and BR insensitive phenotype, as well as down-regulated transcripts of the BR signaling genes and up-regulated BR biosynthesis genes. All these results suggested that brassinosteroid receptor BRI1 is highly conserved among different species, thereby illustrating that *BRI1* gene is essential for plants growth and development in evolution.

A few studies have discussed that BRs effect on the stress tolerance may through the regulation of BR signaling or biosynthesis genes (Divi & Krishna, 2009; Choudhary et al., 2012; Zhao & Li, 2012). The *Arabidopsis* BR insensitive mutant, *bri1*, has an increased tolerance to cold because of the constitutively higher level of expression of *CBFs/DREBs* (Kim et al., 2010). *AtBZR1* can enhance the cold tolerance through the regulation on pectin enthylesterase (PME) activity (Qu et al., 2011). The knockout mutation of rice *OsGSK1*, an ortholog of *AtBIN2*, enhanced tolerance to cold, heat, salt and drought stresses (Koh et al., 2007). These confirmed the important role of BR signaling components playing in stress tolerance. However, the molecular mechanisms through which these BR signaling components interact with stress responsive genes still need to be addressed.

Here we found that knockdown of *BRI1* gene in *Brachypodium* can enhance drought tolerance, which is the first report on temperate grasses. Early studies found that physiological adaptations play a role in elevated drought resistance in plants, for
example reduced cell size in leaves (Henckel, 1964; Hsiao, 1973). The reduction in cell size permits a lower cellular osmotic potential, and hence enhances capacity for turgor maintenance under drought stress (Cutler et al., 1977). In our study, the cell length of RNAi mutant was decreased (Fig. 3d), leading to smaller cell size than wild type, probably giving a lower cellular osmotic potential. Besides, root length is thought as another factor influence the drought tolerance of plant. Longer and deeper roots could give plants better access to water resource available under soil (Sharp & Davies, 1985; Merrill et al., 2002; Moroke et al., 2005). But in our RNAi mutant root length was decreased (Fig. 4e), which is a typical BR insensitive phenotype. The stomatal density was also compared between wild type and RNAi mutants, but statistic analysis showed that there is no significant difference (data not shown). However, overall morphologic changes, such as less tiller number, shorter nodes, smaller leaves, and compact stature (Fig. 3), may confer the enhanced drought tolerance on *BdBRI1* RNAi mutants.

Except the change of morphology, several drought-responsive genes exhibited obvious higher expression than wild type under drought stress (Fig. 7), including *ERD1, RD26* and *P5CS*. Furthermore, *ERD1* and *RD26* were even constitutively expressed in the RNAi mutants. RD26, a NAC (NAM, ATAF1, 2, CUC2) transcription factor, regulated *ERD1* expression through binding to its promoter region (Simpson et al., 2003b; Tran et al., 2004; Tran et al., 2007). Through the ChIP-chip study, it found both *ERD1* and *RD26* are the putative binding targets of the transcription factor BES1 and BZR1 in *Arabidopsis* (Sun et al., 2010; Yu et al., 2011). Moreover, *RD26* was down-regulated in *bzl1-D* overexpression mutant and up-regulated in *bzl1* mutant in
Arabidopsis, suggesting that RD26 was negatively regulated by BES1 and BRI1 (Kim et al., 2010; Sun et al., 2010). In our study, the expression level of BZR1 and BES1 were reduced in the BdBRI1 RNAi mutants, then RD26 would be released from the inhibition of BES1/BZR1 to activate ERD1 leading to increased drought tolerance.

In summary, we identified a BRI1-like gene, BdBRI1, from Brachypodium. The BdBRI1 mutants showed dwarf and BL insensitive phenotypes, corresponding to reduced expression of the BR signaling genes and increased expression of the BR synthesis genes. In addition, increased tolerance to drought stress was found in the RNAi mutants, as well as constitutive expression of drought responsive gene ERD1 and RD26, which are the putative targets of transcription factor BES1 and BZR1. We gave the speculation that the downstream genes of BRI1 such as BES1 or BZR1 exert anti-stress effects on Brachypodium by regulating the transcription factor of the drought-responsive genes or regulating these genes directly. To further elucidate the mechanism of BR signaling on the other stress tolerances in Brachypodium, we are testing the stress responses including cold, heat and salinity in BdBRI1 RNAi mutants as well. Overall, our results gave more evidences that the crosstalk exists between the BR signaling and drought response pathway. Whether there are any other components involved in this crosstalk still need to be studied.
References


Tables

Table S1. Pair-wise sequence identity was calculated between the three \textit{BRII}-like genes, \textit{Bradi}2g48280, \textit{Bradi}4g27440, or \textit{Bradi}3g21400 and BRI1s/BRLs members from different plant species.

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The highlighted number indicated the highest sequence identity between the \textit{BRII}-like genes and other BRI1/BRLs homologs.
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Table S3. BLAST searches of BR-related and drought stress inducible genes in *B.distachyon*

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BLAST searches of *B. distachyon* genome were carried on Phytozome V9.0 server (http://www.phytozome.com/search.php?show=text&method=Org_Bdistachyon) using the query amino acid sequences. The putative genes were isolated with primers designed from the coding region of each gene (Table S2).
Figure legends

Fig. 1 Phylogenetic relationships of BRI1/BRL proteins in various plant species. (a) Partial amino acid alignment of Bradi2g48280 and BRI1s from other species. The underlined amino acid sequences are missing for the monocots. (b) The comparison included the following BRI1 family members: for Arabidopsis, AtBRI1 (AAC49810), AtBRL1 (Q9ZWC8), AtBRL2 (Q9ZPS9), and AtBRL3 (Q9LJF3); for rice, OsBRI1 (BAB68053), OsBRL1 (BAD34326), OsBRL2 (AAK52544), OsBRL3 (BAD01717); for barley, HvBRI1 (BAD06331); for tomato, LeBRI1 (AAN85409), SR160 (Q8GUQ5); for tobacco, NbBRI1 (ABO27628); for pea, PsBRI1 (BAC99050); and for potato, StBRI1 (ABO27627). The phylogenetic tree was constructed using ClustalW with Neighbour Joining method.

Fig. 2 (a) Schematic diagram of the BRI1 RNAi construct. LB - left border, RB - right border, hpt - Hygromycin phosphotransferase gene, 2x35S pro - two copies of the CaMV 35S promoter, SL - stem loop, a inverted repeat fragment from pSR252. nos - the nopaline synthase (nos) terminator. Primers used for screening transgenic plant are indicated with arrows (Table S2). (b) Semiquantitative RT-PCR analysis of the BRI1 expression level in wild type (WT), RNAi-10 and RNAi-27. (c) Real-Time RT-PCR analysis of the BRI1 expression level in the wild type (WT), knockdown mutants of RNAi-10 and RNAi-27. Error bars represent standard error. Differences were considered significant at P<0.05. Levels are not connected by same letter are significantly different.
Fig. 3 (a) Visual appearance of the wild type (left) and RNAi-10 mutant (right) 3 weeks after seed germination. Leaf size of the wild type (left) and RNAi-10 mutant (right). (b) Average tiller number per seedling for the wild type (WT), RNAi-10 and RNAi-27. (c) Average length of an internode for the wild type (WT), RNAi-10, and RNAi-27. Ten seedlings for each line were measured for each experiment. (d) Average cell length of the 4th internode from the wild type and RNAi mutant. (e) Microscopic observation of longitudinal sections of the 4th internode from the wild type and RNAi mutant. Scale bar = 100 µm. Error bars represent standard error. Differences were considered significant at P<0.05. Levels are not connected by same letter are significantly different.

Fig. 4 BL sensitivity test. (a) Lamina inclination response to different concentrations of BL. BL at 0, 10, 100, 1000 ng µl-1 was spotted on the joint between the leaf blade and sheath of the first leaf (from bottom) of one-week old seedlings. Images were taken 24 hours after BL treatment. Seeds were germinated on moisturized filter papers for 1 week in a growth chamber at 23°C. (b) Bending angle in response to different concentrations of BL. The angles were measured by ImageJ software. (c) Coleoptile elongation test. Seeds were germinated on MS media with or without 0.1µM BL for 5 days in the dark at 23°C. Images were taken 5 days after treatment. (d) Average coleoptile length of the wide type (WT) and the RNAi-10 in response to 0.1µM BL. (e) Average root length of the wide type (WT) and the RNAi-10 in response to 0.1µM BL. Error bars represent
standard error. Differences were considered significant at P<0.05. Levels are not connected by same letter are significantly different.

Fig. 5 Real-Time RT-PCR analysis of expression of BR-related genes in the wild type (WT) and the RNAi-10 mutant with or without BL treatment. The expression level of each gene was normalized to that of the BdGAPDH, and levels in WT without BL treatment were set as 1.0. Both WT and the RNAi-10 mutant were sprayed with 10ml of 1μM of BL. Total RNA was prepared from the leaves of seedlings 6 hours after BL treatment, as described in Materials and Methods. Error bars represent standard error. Differences were considered significant at P<0.05. Levels are not connected by same letter are significantly different.

Fig. 6 Enhanced drought stress tolerance in RNAi mutants. (a) After withholding water for 12 days, the wild type plants (left) showed leaf wilting, whereas the RNAi-10 mutants (right) did not show any drought stress symptoms. (b) One week after rewatering, fewer seedlings were recovered in the wild type plants (left) than in the RNAi-10 mutant plants (right). (c) Fv/Fm ratios were measured at the indicated soil moisture level. (d) Leaf water contents (LWC %) were measured when soil moisture content has reached to the 14-15% range. (e) The propagation of plants recovery for the wild type and RNAi-10 mutant after drought stress. Plants were not watered until the soil moisture content reached the 14-15% range when wild occurred and rewatering was done. Data were
recorded one week after rewatering. Error bars represent standard error. Differences were considered significant at $P<0.05$. Levels are not connected by same letter are significantly different.

Fig. 7 Real-Time RT-PCR analysis of the expression of the drought-responsive genes with or without drought treatment. For drought treatment, leaf tissues were harvested when the soil moisture content was between 40-42%. The expression level of each gene was normalized to that of BdGAPDH gene, and the expression level for each gene in WT without drought treatment was set as 1.0. Error bars represent standard error. Differences were considered significant at $P<0.05$. Levels are not connected by same letter are significantly different.

Fig. S1 Comparison of BRI1 amino acid sequences. Deduced amino acid sequences of BRI1 homologs from Arabidopsis, barley, rice, tomato, pea, wheat, potato, and Brachypodium were aligned by using ClustalW program on GenomeNet server (http://www.genome.jp/tools/clustalw/). Phylogenetic analysis was performed by ClustalW with Neighbor Joining method on Phylogeny.fr server (http://www.phylogeny.fr) for the selected BRI1 homologous proteins.

Fig. S2 (b) Semiquantitative RT-PCR analysis of the expression levels of the other two BRI1-like genes in wild type (WT), RNAi-10 and RNAi-27.
Fig. 1
Fig. 2

(a) 

(b) 

(c) 

Fig. 3

(a) 

(b) 

(e) 

(c) 

(d)
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. S1
#### Fig. S1

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Paea/PsBR1 | 250 |
Tomato/SR160 | 260 |
Pimentel11folium/SpB | 270 |
Tomato/LeBR11 | 280 |
Potato/ShBR11 | 290 |
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Pimentel11folium/SpB | 340 |
Tomato/LeBR1 | 350 |
Potato/ShBR11 | 360 |
Rice/OsBR11 | 370 |
Barley/SwBR11 | 380 |
Brad1| 390 |
Arabidopsis/AtBR1L | 400 |
Arabidopsis/AtBRK1L | 410 |
Rice/OsBR1 | 420 |
Rice/OsBR1L | 430 |
Rice/OsBR2 | 440 |
Brad1| 450 |
Rice/OsBR1L | 460 |
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**Consensus** = 50

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Rice/OsBR11 | 300 |
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Brad1| 320 |
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Pimentel11folium/SpB | 340 |
Tomato/LeBR1 | 350 |
Potato/ShBR11 | 360 |
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Barley/SwBR11 | 380 |
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Rice/OsBR2 | 480 |
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Brad1| 500 |
Rice/OsBR2 | 510 |
Arabidopsis/AtBRK2L | 520 |

**Consensus** = 50

| 119 |

Arabidopsis/AtBR11 | 240 |
Paea/PsBR1 | 250 |
Tomato/SR160 | 260 |
Pimentel11folium/SpB | 270 |
Tomato/LeBR11 | 280 |
Potato/ShBR11 | 290 |
Rice/OsBR11 | 300 |
Barley/SwBR11 | 310 |
Brad1| 320 |
Arabidopsis/AtBR1L | 330 |
Pimentel11folium/SpB | 340 |
Tomato/LeBR1 | 350 |
Potato/ShBR11 | 360 |
Rice/OsBR11 | 370 |
Barley/SwBR11 | 380 |
Brad1| 390 |
Arabidopsis/AtBRK1L | 400 |
Rice/OsBR1 | 410 |
Rice/OsBR1L | 420 |
Rice/OsBR2 | 430 |
Brad1| 440 |
Rice/OsBR1L | 450 |
Arabidopsis/AtBRK2L | 460 |
Rice/OsBR2 | 470 |
Rice/OsBR1L | 480 |
Brad1| 490 |
Rice/OsBR2 | 500 |
Arabidopsis/AtBRK2L | 510 |

**Consensus** = 50

| 119 |

Arabidopsis/AtBR11 | 240 |
Paea/PsBR1 | 250 |
Tomato/SR160 | 260 |
Pimentel11folium/SpB | 270 |
Tomato/LeBR11 | 280 |
Potato/ShBR11 | 290 |
Rice/OsBR11 | 300 |
Barley/SwBR11 | 310 |
Brad1| 320 |
Arabidopsis/AtBR1L | 330 |
Pimentel11folium/SpB | 340 |
Tomato/LeBR1 | 350 |
Potato/ShBR11 | 360 |
Rice/OsBR11 | 370 |
Barley/SwBR11 | 380 |
Brad1| 390 |
Arabidopsis/AtBRK1L | 400 |
Rice/OsBR1 | 410 |
Rice/OsBR1L | 420 |
Rice/OsBR2 | 430 |
Brad1| 440 |
Rice/OsBR1L | 450 |
Arabidopsis/AtBRK2L | 460 |
Rice/OsBR2 | 470 |
Rice/OsBR1L | 480 |
Brad1| 490 |
Rice/OsBR2 | 500 |
Arabidopsis/AtBRK2L | 510 |

**Consensus** = 50
Fig. S2
CHAPTER 5 GENERAL CONCLUSION

In this study, we analyzed the expression of VRN genes in *Brachypodium* of either non-vernalization requiring (Bd21) or vernalization requiring (Bd29-1) accession and studied their freezing tolerance. We found that *BdVRN1* and *BdVRN3* were induced by vernalization and were positive regulators for flowering. But *BdVRN2* is likely not involved in the vernalization pathway. Knockdown of *BdVRN1* in Bd21 led to a severe phenotype with delayed flowering, which could be rescued by vernalization treatment. Expression analysis of the *BdVRN1* RNAi mutants showed that *BdVRN1* positively correlated with the expression of *BdVRN3* but had no effect on *BdVRN2*. Meanwhile, enhanced tolerance to freezing stress was observed in the RNAi mutants, accompanied by constitutive expression of several cold responsive genes, including *BdCBF2*, *BdCBF3*, *BdCBF5*, *BdCBF6* and *DREB2A* at high levels. These results suggested that *BdVRN1* plays a critical role on flowering in vernalization pathway, furthermore, acting as a negative signal affecting the regulation of the cold responsive genes.

To further detect the regulation of other cold-responsive genes, we developed a novel method to generate a high-throughput lhRNA expression library from a population of double stranded cDNAs by a Phi29 polymerase amplification mediated method, referred to Phi29-Amplified RNAi Construct (PARC). This method allows the conversion of a cDNA library, especially a tissue- or stress- specific cDNA library, into an lhRNA expression library by one single ligation and an additional cloning step. We generated a suppression subtractive hybridization (SSH) cDNA library from cold treated and non-cold treated *Brachypodium* to focus on transcripts differentially expressed in
response to cold. This library was then converted into an lhRNA expression library for gene silencing. Our results suggested that this novel method provides a high-throughput and reliable platform for making lhRNA interference construct.

To validate the PARC system, a lhRNA construct targeting the brassinosteroids receptor gene BRI1 was tested in Brachypodium. We isolated a BRI1 homolog gene, BdBRI1 from Brachypodium and generated the BdBRI1-RNAi mutants to knock down the BdBRI1 expression level. The RNAi mutants showed a typical BR-insensitive phenotype as dwarfism with compact stature, narrow and short leaf, short internode, decreased BR response, and modulated expression level of BR-related genes. Moreover, we found that knockdown of the BdBRI1 expression conferred highly increased drought tolerance in transgenic plants. Two drought-responsive genes, RD26 and ERD1, were constitutively expressed, which are two putative target genes of transcription factors BES1 and BZR1 (Simpson et al., 2003a; Fujita et al., 2004). To our knowledge, this is the first report of BR signaling pathway in Brachypodium. Therefore, our results not only first confirmed that the BR genes and signaling are conserved between Arabidopsis, rice and Brachypodium, but also showed crosstalk exists between BR signaling pathway and drought stress response in Brachypodium.
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