Establishment of a vernalization requirement in *Brachypodium distachyon* requires *REPRESSOR OF VERNALIZATION1*

Daniel P. Woods[a,b,c], Thomas S. Ream[b,c,1], Frédéric Bouché[c], Joohyun Lee[c,2], Nicholas Thrower[b,d], Curtis Wilkerson[b,d], and Richard M. Amasino[a,b,c,3]

[a]Laboratory of Genetics, University of Wisconsin–Madison, Madison, WI 53706; [b]Department of Energy Great Lakes Bioenergy Research Center, University of Wisconsin–Madison, Madison, WI 53706; [c]Department of Biochemistry, University of Wisconsin–Madison, Madison, WI 53706; and [d]Department of Plant Biology, Michigan State University, East Lansing, MI 48824

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A requirement for vernalization, the process by which prolonged cold exposure provides competence to flower, is an important adaptation to temperate climates that ensures flowering does not occur before the onset of winter. In temperate grasses, vernalization results in the up-regulation of *VERNALIZATION1* (*VRN1*) to establish competence to flower; however, little is known about the mechanism underlying repression of *VRN1* in the fall season, which is necessary to establish a vernalization requirement. Here, we report that a plant-specific gene containing a bromo-adjacent homology and transcriptional elongation factor S-II domain, which we named *REPRESSOR OF VERNALIZATION1* (*RVR1*), represses *VRN1* before vernalization in *Brachypodium distachyon*. That *RVR1* is upstream of *VRN1* is supported by the observations that *VRN1* is precociously elevated in an *rvr1* mutant, resulting in rapid flowering without cold exposure, and the rapid-flowering *rvr1* phenotype is dependent on *VRN1*. The precocious *VRN1* expression in *rvr1* is associated with reduced levels of the repressive chromatin modification H3K27me3 at *VRN1*, which is similar to the reduced *VRN1* H3K27me3 in vernalized plants. Furthermore, the transcriptome of vernalized wild-type plants overlaps with that of nonvernalized *rvr1* plants, indicating loss of *rvr1* is similar to the vernalized state at a molecular level. However, loss of *rvr1* results in more differentially expressed genes than does vernalization, indicating that *RVR1* may be involved in processes other than vernalization despite a lack of any obvious pleiotropy in the *rvr1* mutant. This study provides an example of a role for this class of plant-specific genes.

vernalization | *Brachypodium* | flowering | VRN1 | RVR1

A common adaptation for optimal timing of flowering in temperate climates is the evolution of a vernalization requirement. Vernalization is the process by which plants become competent to flower after prolonged exposure to the cold temperatures of winter (1). Cold exposure alone, however, is typically not sufficient to induce flowering; rather, it often must be followed by an inductive photoperiod, such as the increasing day lengths of spring. The combination of a requirement for a prolonged period of cold followed by a requirement for increasing day lengths has the adaptive value of preventing flowering in the fall season before the onset of winter, which would likely compromise reproductive success (2).

To date, information about the vernalization response in grasses has largely been derived from studies of existing allelic variation in wheat and barley (for reviews, see refs. 3–5). Wheat and barley varieties can be classified as either spring or winter types. Spring varieties do not require vernalization to flower rapidly in inductive long days (LD), whereas vernalization enables rapid flowering of winter varieties (5, 6). A current molecular model of vernalization in temperate grasses consists of a regulatory loop including the genes *VERNALIZATION1* (*VRN1*), *VERNALIZATION2* (*VRN2*), and *VERNALIZATION3* (*VRN3*) (6, 7). *VRN3* is orthologous to *FLOWERING LOCUS T* (*FT*), which encodes a small, mobile, flowering-inducing protein also known as florigen (8, 9); hereafter, *VRN3* will be referred to as *FT1* (10). The regulatory loop is thought to exist in leaves where all three genes are expressed (10–12). In the fall, before cold exposure, high levels of *VRN2* repress *FT1* to prevent flowering (12, 13). *VRN2* contains a putative zinc finger and a CONSTANS, CONSTANS-LIKE, and TIMING OF CAB1 domain and is part of the type VI *CO-like* gene family (12, 14). Before cold exposure, both *VRN1* and *FT1* are expressed at low levels (6). However, during the cold of winter, *VRN1* is up-regulated proportionately to the length of cold experienced, and in turn, higher *VRN1* levels are thought to cause a decrease of *VRN2* expression (11, 15, 16). *VRN1* encodes a MADS box transcription factor related to the *APETALA1*/*FRUITFULL* (*AP1*/*FUL*)-like class of floral homeotic genes in *Arabidopsis thaliana* (17, 18). Like *FUL* in *A. thaliana*, *VRN1* expression is not restricted to the meristem, but it is also expressed in leaves where, in pooid grasses, it participates in the vernalization regulatory loop (19). VRN1 enables *FT1* expression in leaves by turning off *VRN2*. VRN1 has been shown to bind to the promoter of *VRN2* so the repression may be direct (16, 20, 21). Vernalization-mediated

Significance

A key feature in the evolution of all vernalization systems is a cold-regulated component. In pooid grasses, up-regulation of the flowering promoter *VERNALIZATION1* (*VRN1*) by prolonged cold is a key feature of vernalization, although little is known about the genes that repress *VRN1* prior to cold exposure or activate it afterward. Here, we report the identification of *REPRESSOR OF VERNALIZATION1* (*RVR1*), a repressor of *VRN1* that is involved in creating a vernalization requirement in *Brachypodium distachyon*. *RVR1* is present in all sequenced flowering plant genomes but is not found outside the plant kingdom. This report describes a role for the *RVR1* class of genes in plants and an upstream component of the *VRN1* regulatory system.

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Data deposition: The sequences reported in this paper have been deposited in the NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (accession nos. SRX2568123, SRX2568122; rvr1 rep1, SRX2574346; rvr1 rep2, SRX2574345; rvr1 rep3, SRX2574344; Bd213_NV_rep1, SRX2574343; Bd213_NV_rep2, SRX2574342; Bd213_NV_rep3, SRX2574341; Bd213_V_rep1, SRX2574340; Bd213_V_rep2, SRX2574339; and Bd213_V_rep3, SRX2574338).

[1]Present address: Monsanto Company, Chesterfield, MO 63017.

[2]Present address: Biology Department, University of Massachusetts, Amherst, MA 01003.

[3]To whom correspondence should be addressed. Email: amasino@biochem.wisc.edu.

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activation of *VRN1* expression is accompanied by changes in chromatin modifications in a presumed regulatory region of its first intron; specifically, there is a decrease in repressive H3K27 methylation and an increase in the activating H3K4 methylation (22). However, no changes in chromatin modifications have been observed around the *VRN2* locus during or after cold (22). Upon repression of *VRN2*, increasing *FT1* expression contributes to the up-regulation of *VRN1* expression in leaves, creating a positive feedback loop ensuring the inductive flowering signal is maintained (10, 20, 23).

The temperate grass model *Brachypodium distachyon* (*Bd*) (24) contains orthologs of the three vernalization genes *VRN1*, *VRN2*, and *FT1*, which are important in regulating flowering in wheat and barley (25; for review see ref. 26). Furthermore, allelic variation at these genes likely contributes to natural variation in flowering responses in *B. distachyon* because they colocalize under quantitative trait loci controlling flowering-time variation (27, 28). Similar to their role in wheat and barley, *FT1* and *VRN1* are promoters of flowering in *B. distachyon* (29). Overexpression of *FT1* and *VRN1* results in rapid flowering in *B. distachyon* (29) and knockdown of *FT1* and *VRN1* results in delayed flowering (30, 31). As in wheat and barley, *BdVRN1* mRNA levels increase quantitatively during increasing lengths of cold treatment and remain elevated after cold (29, 32, 33). In addition, overexpression of *VRN1* results in elevated expression of *FT1* and overexpression of *FT1* results in elevated *VRN1* expression, consistent with the positive feedback loop suggested from studies in wheat and barley (7, 29). Unlike wheat and barley, however, *BdVRN2* is not suppressed by cold exposure (in fact, *BdVRN2* is induced during the cold) and its expression is not regulated by *VRN1* despite being a LD repressor of flowering (31).

From a screen of ethyl methane sulfonate-treated plants for mutants that flower rapidly without vernalization, we identified *REPRESSOR OF VERNALIZATION1* (*RVR1*), a plant-specific gene containing a bromo-adjacent homology (BAH) and transcriptional elongation factor S-II (TFIIS) domain that is involved in establishing a vernalization requirement in *B. distachyon* by repressing *VRN1* before cold exposure.

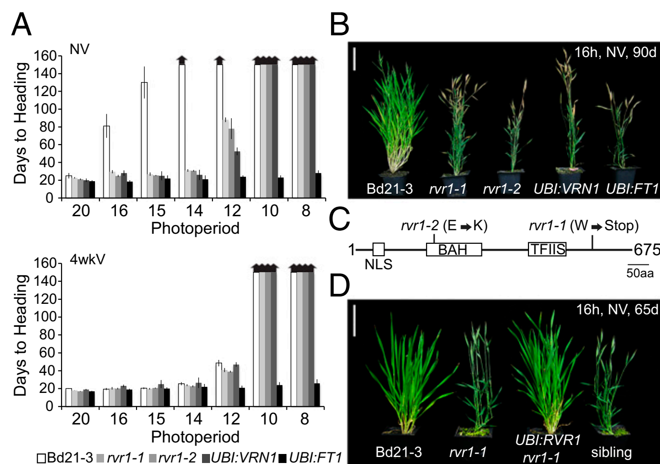

Fig. 1. A gene with a BAH and TFIIS domain (*RVR1*) represses flowering time. (*A*) Flowering times of Bd21-3 wild-type, *rvr1-1*, *rvr1-2*, *UBI:VRN1*, and *UBI:FT1* (*VRN1* and *FT1* cDNA under control of the maize ubiquitin promoter) grown in 20, 16, 15, 14, 12, 10, and 8 h of light either without cold exposure (NV) and with cold exposure (V) for 4 wk (4wkV). Bars represent the average of 12 plants ± standard deviation. The experiment was repeated with similar results. Arrows above bars indicate that none of the plants flowered at the end of the experiment (150 d). (*B*) Representative image of Bd21-3 wild-type, the two alleles of *rvr1*, *UBI:VRN1*, and *UBI:FT1* grown in 16-h of light for 90 d after germination without vernalization (NV). (Scale bar, 5 cm.) (*C*) Domain structure of the 675-aa-long *RVR1* protein showing the location and corresponding amino acid changes of the ethyl methane sulfonate-induced mutations of the two mutant alleles *rvr1-1* and *rvr1-2*. Domain structure of *RVR1* includes a putative nuclear localization signal (NLS), BAH, and TFIIS domains. (*D*) Image of representative T1 plants grown in a 16-h photoperiod. Bd21-3, *rvr1-1*, and segregating non-transgenic sibling plants are controls for the effect of overexpression of the wild-type *RVR1* cDNA in the *rvr1-1* background (*UBI:RVR1::rvr1-1*). (Scale bar, 5 cm.)

Results and Discussion

Isolation of Rapid-Flowering Mutants in *B. distachyon*. To identify genes that are necessary to create a vernalization requirement in temperate grasses, we screened for mutants in *B. distachyon* that flower rapidly without vernalization. From this screen, we identified many rapid-flowering mutants, two of which are allelic (Fig. 1*C*). These two mutants flower rapidly in 16-h daylengths at ∼20 d after germination, with six leaves on the parent culm similar to vernalized wild-type parental Bd21-3 plants, whereas nonvernalized (NV) wild-type [which has a facultative vernalization requirement (29)] flower at around 80–90 d, with greater than 16 leaves on the parent culm (Fig. 1*A*). Interestingly, in 16-h days without vernalization these mutants flower as rapidly as lines constitutively expressing either of two critical floral promoters *BdVRN1* or *BdFT1* (Fig. 1 *A* and *B*). There is no difference in the rate of leaf initiation between the mutant and wild-type in LD or short days (SD). Other than rapid flowering, no other visible mutant phenotypes were observed. Thus, *rvr1* mutants behave as vernalized plants.

Identification of the *RVR1* Gene. To identify the causative lesion in these mutants, we made F2 mapping populations by crossing the mutants, which are in the Bd21-3 background, to the polymorphic mapping partner Bd3-1 [we have previously shown that an F2 derived from crossing the wild-type parents does not segregate for flowering time (34)]. Both mutants were mapped, using previously developed insertion–deletion (indel) markers (34), to the same 400-kb interval around 40 mb on chromosome 2, indicating that they might be allelic. To identify the causal mutation in each mutant line, we carried out whole-genome sequencing on DNA pooled from approximately 100 mutants from the F2 mapping population that segregated for the rapid flowering *rvr1* phenotype. Sequencing reads were analyzed using the CloudMap pipeline (35) as optimized for *B. distachyon* (Fig. S1) (34). The CloudMap results were consistent with our initial indel marker mapping data (Fig. S1), and analysis of the sequence data revealed only one gene in which both mutants contain a lesion. The first allele (*rvr1-1*) contains a C-to-T mutation converting a Trp codon into a premature stop in the last exon in a previously uncharacterized gene (Bradi2g40147), annotated as a gene with a nuclear-localization signal, BAH, and TFIIS domains (Fig. 1*C*). We named this gene *RVR1*. The second allele (*rvr1-2*) contains a C-to-T mutation creating a Glu-to-Lys missense mutation located within the BAH domain (Fig. 1*C*). BAH domains appear to mediate protein–protein interactions in protein complexes involved in chromatin modification or complexes that "read" chromatin states (36–38). The TFIIS domain is associated with transcriptional regulation by interacting with RNA polymerase II in transcriptional elongation (39). qRT-PCR in *rvr1-1* plants revealed a reduction in the abundance of the *RVR1* transcript by nearly 80% compared with wild-type plants, perhaps through nonsense-mediated decay as a result of the presence of the premature stop codon (Fig. S2*A*). *rvr1-2* behaves as a semidominant mutation (for mapping purposes the homozygous mutants are more rapid flowering than heterozygotes). Overexpressing the *RVR1* cDNA using the maize ubiquitin promoter (*UBI:RVR1*) in either *rvr1-1* or *rvr1-2* rescued the mutant phenotype (Fig. 1*D* and Fig. S2*B*).

Phylogenetic analyses of *RVR1* estimate a well-supported clade containing species that span land plant diversification; however, no identifiable orthologs are present outside of the plant kingdom (Fig. S3). Interestingly, *RVR1* does not appear to be part of a gene family; it is found in single copy throughout land plant diversification in the plant genomes queried for this analysis, except

in *B. distachyon*, where there is another related gene, Bradi2g14070, which also has a BAH and TFIIS domain.

To test if the *RVR1* ortholog in *A. thaliana* (*AtRVR1*; At4g11560) affects flowering as it does in *B. distachyon*, we evaluated the phenotype of lines carrying T-DNA insertions in *Atrvr1-1* [*Atrvr1-1* (Salk_017758) and *Atrvr1-2* (Sail_1246_E10)] (Fig. S4*A*). The T-DNA alleles are likely to result in loss-of-function because they are within the coding region of the gene, and semiquantitative PCR revealed the expression of *AtRVR1* was abolished in *Atrvr1-2* (Fig. S4*B*). The mutants flowered the same as wild-type in both 16-h LD and 8-h SD (Fig. S4 *C* and *D*), and we did not observe any visible mutant phenotypes. Additionally, *Atrvr1-2* did not affect flowering after vernalization in a "winter" *A. thaliana* accession that contains a functional *FRIGIDA* (*FRI*) allele (Fig. S4*E*). In *A. thaliana*, FRI is required for the activation of the potent floral repressor, *FLOWERING LOCUS C* (*FLC*) that is repressed by prolonged cold exposure (40, 41). That mutations in *RVR1* do not affect flowering time in *A. thaliana* is not surprising because *A. thaliana* and grass vernalization systems evolved independently (42) and, as discussed below, in *B. distachyon RVR1* acts as a repressor of *VRN1*, but the orthologs of *B. distachyon VRN1* in *A. thaliana* (*AP1*/*FUL*) do not play a role in *A. thaliana* vernalization. Whether a role of *RVR1* in *VRN1* repression is specific to grass vernalization systems or is present more broadly in monocots will be interesting to determine.

Comparison of the *rvr1* Phenotype with Constitutive Expression of *VRN1* and *FT1*. To further characterize the *rvr1* mutant phenotype, we grew wild-type, *rvr1-1*, *rvr1-2*, *UBI:FT1*, and *UBI:VRN1* in different photoperiods (20-, 16-, 15-, 14-, 12-, 10-, 8-h days) either without vernalization (NV) or after a saturating 4-wk vernalization treatment (V) (Fig. 1*A*). NV plants were planted at the end of the vernalization treatment so that V and NV plants were grown at the same time and were developmentally matched. We have previously shown that 12-h daylengths are the shortest daylengths inductive for flowering; shorter daylengths do not permit flowering even in V or *UBI:VRN1* plants (31). Similar to *UBI:VRN1* and V wild-type plants, *rvr1* fails to flower in daylengths less than 12-h. However, 12-h *UBI:VRN1* plants flower ~30–40 d earlier then *rvr1* (Fig. 1*A*). In contrast, *UBI:FT1* plants flower around 20 d with or without vernalization in all of the tested photoperiods, consistent with *FT1* acting downstream of photoperiod sensing (Fig. 1*A*). Thus, *rvr1* is similar phenotypically to V plants and NV plants constitutively expressing *VRN1*.

***VRN1* and *FT1* Expression in *rvr1*.** As discussed above, *rvr1* mutants phenocopy constitutive *VRN1* expression in most daylengths, but in SD-lengths *rvr1* mutants do not phenocopy constitutive *FT1* expression. This finding is consistent with loss of *RVR1* leading to constitutive *VRN1* expression in all daylengths, but not to *FT1* expression in SD. To evaluate this model, we examined *VRN1* and *FT1* mRNA levels in *rvr1* compared with NV wild-type. Indeed, in LD *VRN1* and *FT1* mRNA levels were significantly elevated in *rvr1-1* compared with NV wild-type in both leaves and in shoot tissue enriched for the shoot apical meristem (Fig. 2*A*). Furthermore, when *rvr1* is grown in 8-h SD—a condition in which *rvr1* does not flower rapidly—*VRN1* mRNA expression levels remain elevated despite *FT1* expression being nearly undetectable (Fig. 2*B*). This demonstrates that the up-regulation of *VRN1* in *rvr1* can occur independently of *FT1* expression. This is important to note because a positive feedback loop exists between *VRN1* and *FT1* in *B. distachyon* (29, 32), similar to that reported in wheat and barley (7, 11).

Growth of *rvr1* plants in 12-h days reveals a difference in flowering time between *rvr1* and *UBI:VRN1* (Fig. 1*A*). Specifically, NV *UBI:VRN1* flower ~30–40 d earlier than *rvr1* mutants (Fig. 1*A*). However, this flowering difference is eliminated after vernalization (Fig. 1*A*). One possible explanation for this difference is that the *VRN1* threshold needed to activate flowering is higher in 12-h days versus longer photoperiods; thus, the increased *VRN1* activity when driven by the ubiquitin promoter may achieve that threshold in 12-h daylengths, but loss of *RVR1* may not. Indeed, expression of *VRN1* in *UBI:VRN1* transgenic lines is over 100-fold higher compared with that in *rvr1-1*. Because there is no flowering-time difference between *rvr1* and *UBI:VRN1* after vernalization, we were interested in determining if *VRN1* levels increase in *rvr1* during and after vernalization (Fig. 2 *B* and *C*). We measured

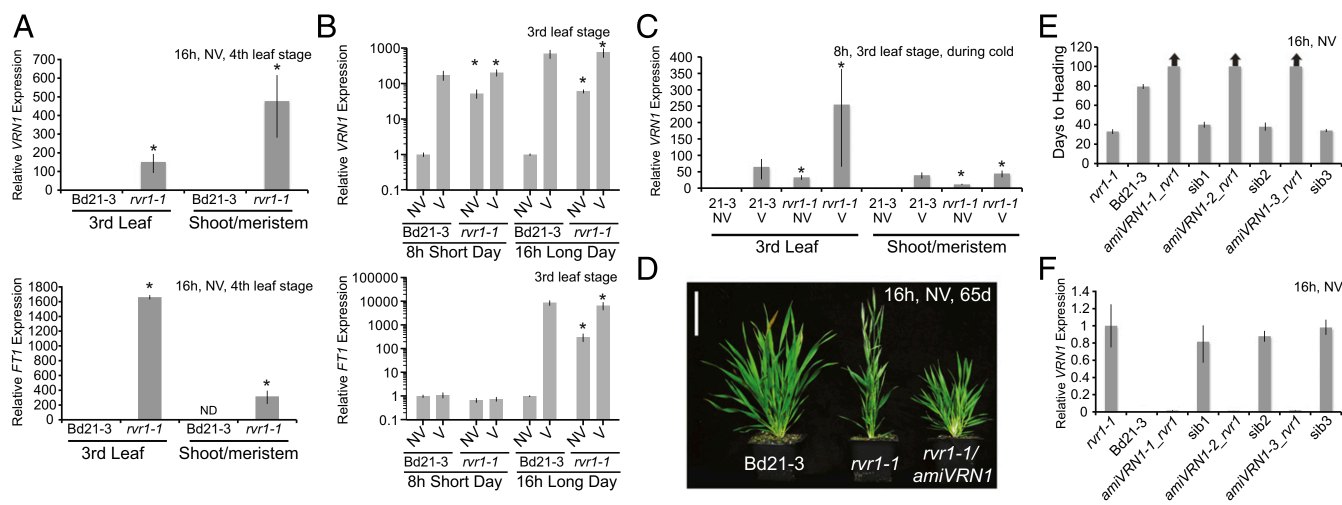

Fig. 2. *RVR1* represses *VRN1* expression and the *rvr1-1* mutant phenotype is dependent upon *VRN1*. (*A*) qRT-PCR data from samples of the third-leaf or meristem-enriched apex (shoot/meristem) of NV Bd21-3 and *rvr1-1* grown in a 16-h photoperiod to the fourth-leaf stage. *VRN1* expression (*Upper*) and *FT1* expression (*Lower*). Bars represent the average of three biological replicates ± standard deviation. (*B*) qRT-PCR of *VRN1* expression (*Upper*) and *FT1* expression (*Lower*). Third leaf harvested at the third-leaf stage of Bd21-3 and *rvr1-1* grown in 8-h SD or 16-h LD. V plants were exposed to 4 wk of cold as an imbibed seed and the NV plants were planted at the end of the vernalization treatment. (*C*) qRT-PCR of *VRN1* expression of leaf three or meristem-enriched shoot apex from Bd21-3 and *rvr1-1* plants either NV or from plants during cold exposure. (*D*) Representative image of Bd21-3 wild-type, *rvr1-1*, and *rvr1-1*/*amiVRN1* grown in 16-h days NV. Image taken 65 d postgermination. (Scale bar, 5 cm.) (*E*) Days to heading of *rvr1-1*, Bd21-3, three independent T1 *amiVRN1*/*rvr1-1* transgenic plants, and nontransgenic sibling plants segregated grown in 16-h days NV. Arrows above bars indicate that none of the plants flowered at the end of the experiment. (*F*) qRT-PCR of *VRN1* expression in *amiVRN1*/*rvr1-1* confirming the knockdown *of VRN1* expression. Asterisks above bars indicate statistically significant contrasts between *rvr1* and Bd21-3 NV (**P* < 0.01).

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VRN1 mRNA levels during cold exposure of seedlings and after vernalization of imbibed seeds (Fig. 2 *B* and *C*). Indeed, *VRN1* mRNA levels in the *rvr1* mutant were elevated by several hundred-fold during and after vernalization (Fig. 2 *B* and *C*). These data are consistent with a threshold model in which *UBI:VRN1* transgenics flower more rapidly than *rvr1-1* in 12-h daylengths because of more highly elevated *VRN1* mRNA levels.

The *rvr1* Rapid-Flowering Phenotype Is Dependent upon *VRN1*. To determine whether the alteration of *VRN1* expression is required for the *rvr1* phenotype, we knocked-down *VRN1* expression using an artificial microRNA that is specific to *VRN1* and does not affect the expression of the closely related *FUL2* paralog (31) (*amiVRN1*) in the *rvr1-1* background (Fig. 2 *D* and *E*). *amiVRN1* delays flowering in wild-type Bd21-3 even after vernalization (31). The *amiVRN1* suppressed the *rvr1-1* phenotype in all 33 T0 transgenic plants we generated; these transgenic lines took longer than 200 d to flower, even under highly inductive 20-h days (29). Furthermore, we also characterized the progeny of three independent T0 lines; those with the *amiVRN1* construct were delayed in flowering, whereas sibling plants lacking the transgene exhibited the rapid flowering *rvr1* phenotype (Fig. 2*E*). qRT-PCR confirmed that the delayed-flowering phenotype was associated with reduced *VRN1* expression in the *rvr1-1* background because *VRN1* expression in *amiVRN1*/*rvr1* was significantly lower than in sibling plants lacking the transgene (Fig. 2*F*). Thus, the rapid flowering *rvr1* mutant is dependent upon *VRN1*.

Expression of Other Putative Flowering Genes in *rvr1*. Expression of other putative flowering-time genes are also elevated in *rvr1*. The *FT1* and *VRN1* paralogs, *FUL2* and *FT2*, are up-regulated in the *rvr1-1* background compared with wild-type (Figs. S5 *A* and *B* and S6 *A* and *B*). No significant expression differences of the flowering-time repressor *VRN2* were observed between wild-type and *rvr1-1* in leaf and meristem tissues (Fig. S5); however, under 8-h SD *VRN2* mRNA levels were somewhat elevated in *rvr1-1* compared with wild-type (Fig. S6).

mRNA levels of two *FLC*-like genes, *OS2* and *MADS37* (43), were also elevated in *rvr1-1* compared with wild-type (Fig. S5 *E* and *F*). As noted above, in *A. thaliana FLC* is a potent flowering repressor that confers a vernalization requirement in Brassicaceae and is repressed by cold (2). The up-regulation of *OS2* and *MADS37* expression in *rvr1-1* is not likely to be a result of cold-mediated *VRN1* expression because in lines overexpressing *VRN1*, *MADS37*, and *OS2* mRNA levels do not change (31).

***RVR1* Is Constitutively Expressed and Its Overexpression Does Not Suppress the Vernalization Response.** Because *RVR1* is involved in the repression of *VRN1* before vernalization, we evaluated whether or not the promotion of flowering by vernalization might involve modulation of *RVR1* expression. We found, *RVR1* transcript levels were not affected by vernalization (Fig. S7). Additionally, we tested if *RVR1* mRNA levels are affected by photoperiod and found that *RVR1* mRNA levels were not significantly different under 8-, 16-, or 20-h photoperiods (Fig. S7).

We also analyzed *RVR1* mRNA levels in different accessions to determine if there was a correlation with flowering time in a 20-h photoperiod (a condition in which rapid-flowering accessions have elevated *VRN1* expression compared with delayed-flowering accessions). *RVR1* expression varied among accessions by less than twofold and there was no consistent pattern of *RVR1* expression level between the rapid flowering or the delayed flowering accessions (Fig. S7).

The rapid-flowering phenotype of the *rvr1* mutant indicates that *RVR1* acts as a repressor of flowering. To test if increased expression of *RVR1* might delay flowering or prevent vernalization, we grew NV and V *UBI:RVR1* transgenics in a 20-h photoperiod and measured days to heading (Fig. S2). *UBI:RVR1* transgenic plants flowered approximately at the same time as wild-type, with or without vernalization (Fig. S2). Thus, the *RVR1* expression level is not likely to be limiting in flowering repression in *B. distachyon*.

Modulation of *VRN1*, *FT1*, and *VRN2* Expression Does Not Affect *RVR1* mRNA Levels. To test if *VRN1*, *FT1*, or *VRN2* affects *RVR1* expression, we measured *RVR1* mRNA levels in *UBI:VRN1*, *UBI:FT1*, *UBI:VRN2*, *amiVRN2*, and *amiVRN1* transgenic plants. In all cases, *RVR1* expression was not significantly affected in these different genetic backgrounds (Fig. S8). Thus, these genes do not appear to be involved in a feedback loop with *RVR1*, and this is consistent with *RVR1* being upstream of these genes in the vernalization pathway (Fig. S8).

The *rvr1-1* Mutant Exhibits Reduced Levels of H3K27me3 Around the *VRN1* Locus Similar to Vernalized Samples of Bd21-3. In barley, induction of *HvVRN1* by vernalization correlates with a reduction of the repressive chromatin modification trimethylation at lysine 27 of histone 3 (H3K27me3) (22). Specifically, repression of *HvVRN1* before vernalization is associated with high levels of H3K27me3, and during vernalization the level of H3K27me3 decreases close to the transcription start site of *HvVRN1* and within its first intron (22). The stable reduction of H3K27me3 after vernalization at the *HvVRN1* locus has been suggested to contribute to the stable transcriptional "on state" of *HvVRN1* after vernalization (22), and this stable on state may contribute to the memory of a vernalized state in cereals (22, 32). Indeed, that *BdVRN1* expression in *B. distachyon* is also maintained after cold exposure in SD conditions, in which *FT1* is not expressed and flowering does not occur, indicates that vernalization is likely to cause a stable *BdVRN1* on state more broadly throughout Pooideae (32).

We find that in NV, wild-type Bd21-3 control seedlings grown in SD, the levels of H3K27me3 are reduced at *BdVRN1* after vernalization as in barley (Fig. 3), and this correlates with the stable induction of *BdVRN1* expression observed after vernalization (32). The precocious *VRN1* expression observed in NV *rvr1-1* mutants (Fig. 2 *A* and *B*) is also associated with decreased levels of H3K27me3 relative to NV Bd21-3 control samples (Fig. 3 *B–E*). Interestingly, at all four sites tested within the *VRN1* locus, H3K27me3 levels in NV *rvr1-1* are similar to those in V Bd21-3 samples (Fig. 3 *B–E*). Thus, RVR1 is required to achieve the levels of H3K27me3 associated with the repressed state of *BdVRN1* in wild-type before vernalization.

Transcriptomics in the *rvr1* Mutant and Vernalized Plants. To explore the specificity of *RVR1* in establishing a vernalization requirement, we performed RNA sequencing followed by transcriptomic analyses in the NV *rvr1-1* mutant versus V and NV wild-type plants. To avoid the confounding effects of flowering, RNA was prepared from plants that were grown in 8-h SD. To focus on genes that were stably affected by vernalization, RNA was prepared from V plants 3 wk after cold exposure. We found 575 genes differentially expressed in newly expanded third-leaf tissue in NV *rvr1-1* compared with NV wild-type plants [fold-change (FC) > 2; $P < 0.001$] (Fig. 4). The enrichment of up-regulated genes in *rvr1-1* (58%) is consistent with an *RVR1* repressor function (Fig. 4*A*). In V plants, 83 genes were differentially expressed compared with NV controls (FC > 2; $P < 0.001$) (Fig. 4). *VRN1* was one of most highly up-regulated genes in *rvr1-1* and the most highly up-regulated gene in V plants (Fig. 4*B* and Fig. S9). The strong and stable induction of *VRN1* by cold supports the important role of *VRN1* in the maintenance of the vernalized state (32). Overall, 10 of the 15 (66%) up-regulated genes and 37 of the 68 (54%) down-regulated genes in V plants overlap with genes up or down-regulated in *rvr1* mutants, consistent with loss of *RVR1* creating at least a partial mimic of a V state at a molecular level (Fig. 4). Furthermore, many other differentially expressed genes that did not exceed the FC

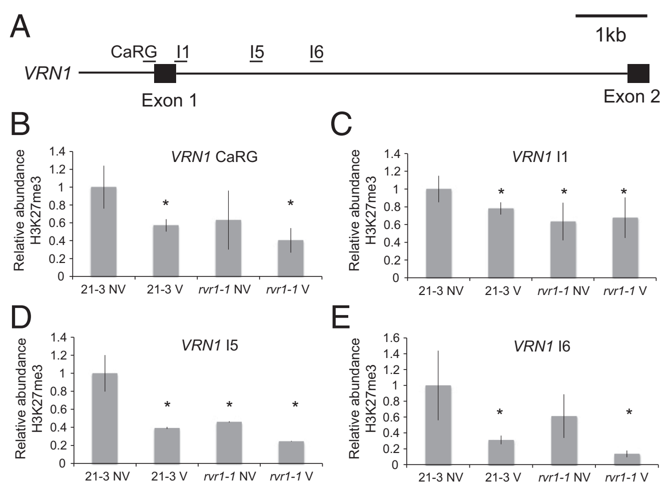

Fig. 3. The effect of vernalization on the histone modification, H3K27me3 at *BdVRN1* in Bd21-3 and *rvr1-1* seedlings. (*A*) Diagram of the 5′ end of *BdVRN1* showing the regions analyzed by ChIP, followed by qRT-PCR. (*B*–*E*) Relative abundance of H3K27me3 at *BdVRN1* in NV and V seedlings from Bd21-3 and *rvr1-1*. Data represent the mean ± SEM from three biological replicate experiments. An asterisk (*) indicates significant differences compared with 21-3 NV (*$P < 0.05$). Primer sequences are provided in Table S1.

threshold discussed above also showed a similar trend in V plants (Fig. 4*B* and Fig. S9). However, 528 additional genes are differentially expressed in the *rvr1-1* mutant compared with the V transcriptome, indicating that *RVR1* is a more general regulator not specific to vernalization (Fig. 4*A*). Although a broader role for *RVR1* might predict that loss of *RVR1* activity would deleteriously affect other plant processes, we did not observe any visible phenotypes other than an effect on flowering in the *rvr1* mutant. However, the plants were well-fertilized and grown in a growth chamber, conditions in which other phenotypes might not be apparent.

Concluding Remarks

RVR1 is required for the repression of *VRN1* before cold. Thus, *RVR1* plays a role in establishing a vernalization requirement in *B. distachyon* and is likely to play the same role in other vernalization-requiring pooid grasses. It is also interesting that although *RVR1* is a plant-specific gene that is conserved across the plant kingdom, this work is a unique reported example of a phenotype due to loss of *RVR1*.

There are several possibilities as to how *RVR1*-mediated repression of *VRN1* is overcome by exposure to prolonged cold, enabling the achievement of a vernalized state. One possibility is that the capability for *RVR1* repression is maintained after vernalization, but *VRN1* activators are induced by cold that outcompete *RVR1*-mediated repression. Another possibility is that *RVR1* activity is abolished by cold. *RVR1* is constitutively expressed, and its expression is not influenced by cold or daylength; however, a vernalization-mediated direct inactivation of RVR1 could occur via a posttranscriptional mechanism, such as differential translation, protein modification, or protein turnover. However, a direct inactivation may be unlikely because transcriptional profiling reveals that *RVR1* affects the expression of many more genes than does vernalization: that is, *RVR1* appears to have a broader role than simply to create a vernalization requirement, and thus loss of *RVR1* activity might deleteriously affect other plant processes. Alternatively, RVR1 could be part of a general complex with different permutations, and loss of a flowering-specific component of a *VRN1*-repressing permutation after prolonged cold exposure could abolish *VRN1* repression. An example in which a specific permutation of a general chromatin-modifying complex provides flowering specificity exists in *A. thaliana*. The polycomb repressive complex 2 (PRC2) is involved in the repression of many *A. thaliana* genes, but during vernalization a subset of the PRC2s acquires the vernalization-specific component *VERNALIZATION INSENSITIVE 3*, which is necessary for PRC2 to regulate *FLC* and achieve a vernalized state (44, 45).

Materials and Methods

Mutant Screen and *RVR1* Gene Identification. The mutant screen was conducted as previously described (34). Mutagenized plants were imbibed for 3 d at 5 °C before being placed into soil and grown under 16-h days at 21 °C. *rvr1-1* was backcrossed three times to Bd21-3 to reduce background mutations and the backcrossed material was used in all experiments. The *rvr1* mutation was mapped using the CloudMap pipeline optimized for *B. distachyon*, sequence data can be found at the National Center for Biotechnology Information (NCBI) Sequence Read Archive rvr1-1, SRX2568123; rvr1-2, SRX2568122 (34).

Plant Growth and Flowering-Time Measurements. Growth and scoring of plants were done as described in Ream et al. (29). Differences in heading date and gene expression between wild-type, nontransgenic, and transgenic plants were assessed using the Student's *t* test and deemed significant if $P < 0.05$.

Phylogenetic Analysis. Phylogenetic analyses were performed using both the full-length *BdRVR1* gene and the individual BAH or TFIIS domains as seed sequences for BLAST searches using Phytozome, CoGe, and the NCBI (46). Handling of sequence and Bayesian trees were generated as described by Woods et al. (46). Maximum-likelihood analyses were conducted using SeaView v4.5.4 (47).

qRT-PCR. RNA extraction and expression analysis was done as detailed in Ream et al. (29).

Generation of UBI:RVR1. For generation of UBI:RVR1, see *SI Materials and Methods*.

Generation of amiVRN1/rvr1-1 Transgenic Lines. The *amiVRN1* construct used to knock-down *VRN1* expression in *rvr1-1* was previously published (31). Transformation of embryonic tissue was conducted as done in Ream et al. (29).

ChIP:H3K27me3. Wild-type and *rvr1-1* were grown to the third-leaf stage in 8-h SD. Eight grams of above-ground seedling tissue (four replicates) were harvested into liquid nitrogen in the middle of the photoperiod and stored at −80 °C. The remaining seedlings were moved to a 5 °C cold chamber set for 8-h SD and were vernalized for 4 wk, which is a saturating vernalization treatment for Bd21-3 (29). After 4 wk of cold exposure, the seedlings were still at

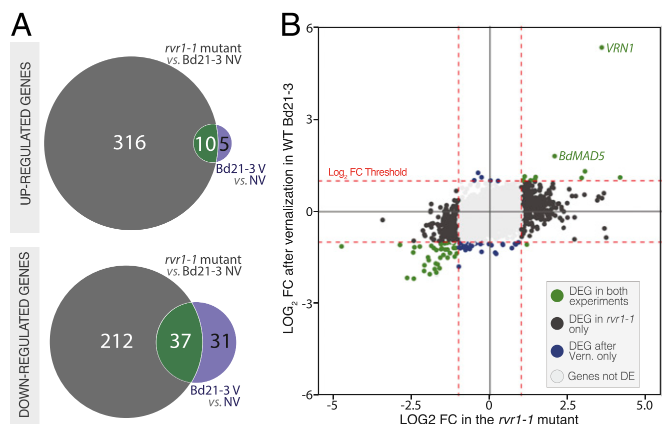

Fig. 4. Effects of *rvr1-1* mutation and vernalization on the leaf transcriptome. (*A*) Overlap between the transcriptomic changes observed in *rvr1-1* seedling and Bd21-3 wild-type plants postvernalization (FC > 2; $P < 0.001$). (*Upper*) Number of genes up-regulated; (*Lower*) number of genes down-regulated. (*B*) Comparison of the FC observed in the nonvernalized *rvr1-1* mutant and in vernalized Bd21-3 seedlings. Red dotted lines indicate fold-change thresholds used for the identification of differentially expressed genes (DEG).

the third-leaf stage and 8 g of above-ground portion of the seedling were harvested into liquid nitrogen during the middle of the photoperiod and stored at –80 °C. For isolation of chromatin and ChIP details, see *SI Materials and Methods*.

RNA-seq Analysis. See *SI Materials and Methods* for plant growth and RNA-seq analysis details. Also see Dataset S1 for a complete list of differentially expressed genes, which shows the FC in a logarithmic scale [$\log_2$(FC)]. Note that genes were considered as differentially expressed when FC > 2 [i.e., $\log_2(FC) > 1$] and adjusted $P < 0.001$.

For RNA-seq validation, the expression of four differentially expressed genes from the RNA-seq analysis were randomly chosen for qPCR analysis to independently validate the RNA-seq analysis (Fig. S9). Plant growth conditions and harvesting procedure were the same as described above; however, the validation was conducted with a distinct set of biological replicates. The expression of all of the genes tested was consistent with the RNA-seq analysis (Fig. S9).

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