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Permalink
https://escholarship.org/uc/item/7w98n2p1

Journal
PLoS genetics, 8(12)

ISSN
1553-7390

Authors
Chen, Andrew
Dubcovsky, Jorge

Publication Date
2012

DOI
10.1371/journal.pgen.1003134

Peer reviewed
Wheat TILLING Mutants Show That the Vernalization Gene VRN1 Down-Regulates the Flowering Repressor VRN2 in Leaves but Is Not Essential for Flowering

Andrew Chen¹, Jorge Dubcovsky¹,²,³*

¹ Department of Plant Sciences, University of California Davis, Davis, California, United States of America, ² Howard Hughes Medical Institute, Chevy Chase, Maryland, United States of America, ³ Gordon and Betty Moore Foundation, Palo Alto, California, United States of America

Abstract

Most of the natural variation in wheat vernalization response is determined by allelic differences in the MADS-box transcription factor VERNALIZATION1 (VRN1). Extended exposures to low temperatures during the winter (vernalization) induce VRN1 expression and promote the transition of the apical meristem to the reproductive phase. In contrast to its Arabidopsis homolog (APETALA1), which is mainly expressed in the apical meristem, VRN1 is also expressed at high levels in the leaves, but its function in this tissue is not well understood. Using tetraploid wheat lines with truncation mutations in the two homoeologous copies of VRN1 (henceforth vrn1-null mutants), we demonstrate that a central role of VRN1 in the leaves is to maintain low transcript levels of the VRN2 flowering repressor after vernalization. Transcript levels of VRN2 were gradually down-regulated during vernalization in both mutant and wild-type genotypes, but were up-regulated after vernalization only in the vrn1-null mutants. The up-regulation of VRN2 delayed flowering by repressing the transcription of FT, a flowering-integrator gene that encodes a mobile protein that is transported from the leaves to the apical meristem to induce flowering. The role of VRN2 in the delayed flowering of the vrn1-null mutant was confirmed using double vrn1-1vrn2- null mutants, which flowered two months earlier than the vrn1-null mutants. Both mutants produced normal flowers and seeds demonstrating that VRN1 is not essential for wheat flowering, which contradicts current flowering models. This result does not diminish the importance of VRN1 in the seasonal regulation of wheat flowering. The up-regulation of VRN1 during winter is required to maintain low transcript levels of VRN2, accelerate the induction of FT in the leaves, and regulate a timely flowering in the spring. Our results also demonstrate the existence of redundant wheat flowering genes that may provide new targets for engineering wheat varieties better adapted to changing environments.

Introduction

The temperate grasses, which include economically important species such as wheat, barley, rye, and oats, are well-adapted to cold winters. Most of these species require a prolonged period of cold treatment for timely flowering, a process referred to as vernalization. This requirement delays the initiation of the reproductive phase and protects the sensitive floral meristems from frost damage during the winter. It also contributes to the precise adjustment of flowering time to seasonal changes, which is important to maximize seed production. Therefore, a better understanding of the mechanisms involved in the regulation of wheat flowering can contribute to the engineering of high yielding varieties adapted to changing environments.

The cloning of the three main wheat vernalization genes, VRN1 [1–3], VRN2 [4] and VRN3 [5], and the characterization of their natural allelic variation [6–11] provided an important first step in our understanding of this regulatory pathway. However, the mechanisms involved in the interactions among these genes are still controversial [12–14]. To facilitate the discussion of these complex interactions, the information available for these three regulatory genes is presented first.

The VRN3 gene is the main integrator of the vernalization and photoperiod signals in the temperate grasses [15] (Figure 1). This gene encodes a RAF kinase inhibitor–like protein with high similarity to Arabidopsis protein FLOWERING LOCUS T (FT) [5] and will therefore, be designated as FT hereafter. In Arabidopsis, FT transcription is induced by long days in the leaves and the encoded protein travels through the phloem to the stem apical meristem [16]. There, FT interacts with the bZIP transcription factor FD and up-regulates the expression of the meristem identity gene APETALA1 (AP1), which leads to the transition of the stem apical meristem from the vegetative to the reproductive phase [17]. A similar interaction has been observed in wheat, where the homologous FT protein interacts with an FD-like protein (FDL2) that has the ability to bind in vitro the promoter of VRN1, the wheat homolog of AP1 [18] (Figure 1).

The insertion of a repetitive element in the FT promoter in the wheat variety Hope results in the overexpression of FT and early flowering. Transformation of a winter wheat with this FT allele
Author Summary

Crop yields are strongly associated with flowering time, therefore a precise understanding of the mechanisms involved in the regulation of flowering is required to engineer varieties adapted to new or changing environments. In wheat, most of the natural variation in flowering time is determined by VERNALIZATION1 (VRN1), a gene responsible for the transition of the apical meristem from the vegetative to the reproductive phase. Extended exposures to low temperatures during winter (vernalization) induce VRN1 expression, which promotes flowering in the spring. VRN1 is expressed in the apices and in the leaves, but its role in the leaves is not well understood. Using two sets of VRN1 knock-out mutants, we demonstrate that a central role of VRN1 in the leaves is to maintain low transcript levels of the VRN2 flowering repressor, which allows the production of the mobile FT protein (florigen) required to initiate flowering. Both sets of VRN1 knock-out mutants flowered very late but, eventually, produced normal flowers and seeds, which demonstrates that VRN1 is not essential for wheat flowering. This last result also demonstrates the existence of redundant flowering genes that could provide new targets for engineering flowering time in wheat.

results in accelerated flowering even in the absence of vernalization, which suggests that high FT transcript levels are sufficient to overcome the vernalization requirement [5]. Furthermore, transcript levels of different wheat and barley FT alleles correlate well with flowering time, which suggests that the amount of FT transcript in the leaves is critical for the regulation of flowering time in the temperate cereals [5].

FT and upstream genes of the photoperiod pathway are well conserved between Arabidopsis and the temperate cereals, but the vernalization genes in these species are very different. The main Arabidopsis vernalization genes FLOWERING LOCUS C (FLC) and FRIGIDA (FR) have not been detected in the temperate cereals, and similarly, the central flowering repressor VRN2 has not been detected in Arabidopsis [4]. Despite the fact that they belong to different classes of proteins, VRN2 and FLC both repress FT and prevent flowering until the plants are vernalized (Figure 1) [5,19]. Recent studies suggest that the negative regulation of FT transcription by VRN2 in the temperate cereals is mediated by the competition between VRN2 and the photoperiod protein CONSTANS (CO, a promoter of FT expression) for binding with a common set of NF-Y transcription factors [15] (Figure 1). NF-Y transcription factors have been shown to be involved in FT activation in Arabidopsis [20,21].

The VRN2 locus includes two tandemly duplicated CCT domain (CONSTANS, CO-like, and TOC1) genes, CCT1 and CCT2, which function as long day flowering repressors [4]. Simultaneous deletions or non-functional mutations in all CCT genes result in a spring growth habit in both barley and wheat [4,11]. In the commercial tetraploid wheat varieties studied thus far (including the variety ‘Kronos’ used in this study), CCT-A1, CCT-A2, and CCT-B1 all have natural deleterious mutations in the CCT domain and the only functional copies are the two similar CCT-B2 genes present in the VRN2-B2 locus [11]. Therefore, a natural deletion including both CCT-B2 genes was sufficient to generate a tetraploid wheat with spring growth habit [11]. Indirect evidence suggests that VRN2 transcription is repressed in the spring by the up-regulation of VRN1, closing a positive feedback regulatory loop that is central for the precise regulation of flowering time in the temperate cereals (Figure 1) [22].

The wheat VRN1 gene encodes a MADS-box transcription factor closely related to the three paralogous Arabidopsis meristem identity genes AP1, CAULIFLOWER (CAL), and FRUITFULL (FUL) [1,3]. VRN1 transcripts are significantly up-regulated during vernalization, both under long and short days. Since FT and VRN2 transcript levels are undetectable under short days [23,24], it was concluded that VRN1 is a direct target of vernalization. This conclusion agrees with the observation that vernalization promotes an active chromatin state by increasing levels of histone 3 lysine 4 trimethylation (H3K4me3) and decreasing H3K27me3 in VRN1 regulatory regions but not in those of VRN2 or FT [25].

In the temperate cereals there are two additional MADS-box genes similar to VRN1 designated as FUL2 (= HvMADS8 = Os-MADS15) and FUL3 (= HvMADS3 = OsMADS18) [26]. The duplications that gave rise to these three paralogous genes in wheat are independent from the duplications in Arabidopsis since they occurred after the monocot-dicot divergence [1]. Therefore, the sub-functionalization of the duplicated meristem identity genes was also independent in these two lineages. In Arabidopsis, AP1 and CAL transcripts are mostly confined to the developing flowers [27] whereas FUL transcripts are detected both in apices and leaves but at low levels [28]. In contrast, high levels of VRN1 have been observed in the leaves of the temperate grasses before the emergence of spikelet primordia, which suggests that VRN1 is part of an early signal involved in the transition from the vegetative to reproductive stages [1,29,30].

In Arabidopsis, all three paralogs have retained meristem identity functions, and only the triple ap1-cal-ful mutant is unable...
to form flowers [31]. In rice, in addition to the AP1/FUL2/FUL3 homologs a fourth MADS-box gene (OsMADS34 = PAP2) needs to be deleted to abolish the transition of the shoot apical meristem to the reproductive stage [32]. In contrast, diploid wheat radiation mutants lacking the VRN1 gene are unable to flower, which suggests that VRN1 is essential for wheat flowering [33]. These mutants, designated maintained vegetative phase (mvp), showed an unexpected down-regulation of FT transcript levels [13], that was not predicted by current flowering models [Figure 1]. However, a more recent study showed that the radiation deletions that eliminated VRN1 were much larger than originally described and included the wheat ortholog of AP1 as well as several other linked genes. Therefore, the mvp mutant results are open to alternative interpretations that are critical for understanding flowering models in temperate grasses [13,14].

To determine the specific role of VRN1 in the induction of flowering and in the regulation of downstream flowering genes we developed truncation mutants for the A and B genome copies of VRN1 in tetraploid wheat (henceforth, Δvrn-A1 and Δvrn-B1) and combined them to generate two sets of mutants with no functional copies of VRN1 (henceforth, Δvrn-null). Using these mutants we demonstrate that functional VRN1 proteins are not essential for wheat flowering or for the up-regulation of FT. We also show that VRN1 expression in the leaves is important for maintaining low transcript levels of VRN2 in the leaves after vernalization, which is critical for a timely flowering in the spring.

**Results**

**Generation of VRN1 and VRN2 mutant combinations.**

*Screening.* The Kronos tetraploid cultivar used to develop the TILLING population carries a functional VRN-A1 allele, which is defined by a large deletion in intron one associated with spring growth habit. Kronos VRN-B1 allele has no intron one deletion and is responsive to vernalization [7]. As described in the introduction, the two closely related ZZCCT-B2 genes (VRN-B2 locus) are the only functional ZZCCT copies in Kronos [11]. Since the VRN-A1 allele for spring growth habit is dominant and epistatic to VRN2, Kronos has a spring growth habit. This genotype will be referred to as “wild type” in this study.

The screening of the tetraploid Kronos TILLING population [34] with primers described in Table S1 yielded 33 mutations for the VRN-A1 gene (Δvrn-A1) and 44 mutations for the VRN-B1 gene (Δvrn-B1). Mutations resulting in truncations or amino acid changes are described in Table S2. Two mutations resulting in either a premature stop codon or the removal of a splice site were selected for each VRN1 homolog (Figure S1A). For each of these mutations we recovered homozygous lines, which all show a single chromosomal peak at the mutant site (Figure S1B). This result demonstrates that Kronos has a single copy of each of the VRN1 homologs.

**Selected mutations.** Mutants Δvrn-A12235 and Δvrn-B12514 have premature stop codons in exons three and four. These mutant alleles encode VRN1 proteins that lack approximately half of the conserved K-box (45% to 66%, respectively) and the complete activation domain (Figure S1A). Sequencing of the Δvrn-A12235 transcripts revealed that the mutation at the acceptor splice site of intron two (AG) to (AA) resulted in the utilization of the mutated base and the adjacent G in exon three as a new acceptor splice site. This 1-bp frame shift mutation resulted in an altered protein sequence with a premature stop after the first nine amino acids. The mutations at the donor splice site of intron one (GT to AT) in Δvrn-B12619 resulted in an immediate stop codon. Transcripts from this VRN-B1 allele were not detected in the expression experiments (PCR primers in exon junction 5–6 and exon 9 [5]), likely due to the long (~10 kb) un-spliced intron. Proteins encoded by the two splice site mutants are predicted to have truncations including 84% and 100% of the conserved K-box and the complete activation domain. In summary, the large truncations predicted in the four VRN1 mutant proteins are almost certain to knock-out its normal function.

**Combination of different mutations.** The individual mutants were backcrossed once to the non-mutagenized Kronos to reduce background mutations and were then inter-crossed to produce two independent mutants with no functional copies of VRN1 (Δvrn-null). The Δvrn-null set 1 was derived from the stop codon mutants Δvrn-A12235 and Δvrn-B12514, and the Δvrn-null set 2 from the splice site mutants Δvrn-A12619 and Δvrn-B12619 (Figure S1).

The VRN1 mutations from the Δvrn-null set 2 were also combined with mutations in the VRN2 genes from a Δvrn-null tetraploid mutant identified in a previous study [11]. The Δvrn-null line carries a deletion encompassing all ZZCCT genes at the VRN-B2 locus in addition to the natural mutations present in most durum varieties that cause non-functional amino acid substitutions at conserved positions of the CCT domains of the ZZCCT-A1 and ZZCCT-A2 genes (VRN-A2 locus) [11]. The Δvrn-null mutant was backcrossed twice to Kronos and then inter-crossed with the Δvrn-null to generate a line lacking functional copies of both VRN1 and VRN2 genes, which is designated hereafter as Δvrn-Δvrn-null. A sister line carrying the functional vernalization-insensitive VRN1 allele (ΔvrnB1-Δvrn-null, spring growth habit) was also selected to be used as control in the vernalization experiments including the Δvrn-Δvrn-null mutants. Table 1 summarizes the vernalization responses and the VRN1 and VRN2 functional and non-functional alleles present in each mutant.

**Effects of VRN1 mutations on heading time and flower morphology.**

*Single gene mutants.* The unvernalized Δvrn-B1 mutants (Δvrn-B1 set 1 and Δvrn-B1 set 2) flowered as early as the Kronos wild type plants (P = 0.38, 51–56 d, Figure 2B). These mutants have a functional VRN-A1 gene with a large deletion in intron one, which results in its expression to high levels even in the absence of vernalization [7]. These lines are insensitive to vernalization and were used as controls to adjust heading times of the vernalized plants in their respective mutant sets in Figure 2A (black columns, see Material and Methods).

The unvernalized Δvrn-A1 mutants flowered 96 (Δvrn-A1 set 1) and 143 (Δvrn-A1 set 2) days later than the Δvrn-B1 mutants (Figure 2A). This is expected since these lines have a functional VRN-B1 allele that is known to be responsive to vernalization [7]. Vernalization accelerated heading time of both Δvrn-A12235 and Δvrn-A12619 by 84 and 133 days respectively (Figure 2A). These results confirmed that the spring growth habit of Kronos is determined by the VRN-A1 allele and that the VRN-B1 allele is responsive to vernalization.

*Δvrn-null mutants.* In the absence of vernalization, the two sets of Δvrn-null mutants headed 11–15 days later than the Δvrn-A1 single mutants but the differences were not significant (Figure 2A, P > 0.05). However, when these plants were vernalized, the Δvrn-null mutants flowered 62 and 113 days later than the corresponding Δvrn-A1 mutants (Figure 2A, P < 0.0001), which indicates that the lack of functional VRN1 proteins in the Δvrn-null mutants greatly reduced their ability to respond to vernalization. This response, however, was not completely eliminated, since vernalized Δvrn-null mutants still flowered 37 days (P = 0.005, set 1) and 31 days (P = 0.035, set 2) earlier than the unvernalized Δvrn-null mutants (Figure 2A). This result was
further confirmed in an independent experiment, in which the vernalized \(\text{vrn1-} \) null mutants flowered 27 days earlier than the unvernalized ones (set 2, Figure 2C).

Both vernalized and unvernalized \(\text{vrn1-} \) null mutants produced normal flowers and fertile seeds (Figure 2B, bottom panel), which indicates that \(\text{VRN1} \) is not essential for the initiation of flowering or for floral and seed development in wheat. These results also indicate that there are redundant flowering genes with functions overlapping those of \(\text{VRN1} \).

**\(\text{vrn1-} \) \text{vrn2-null} \) mutants.** To determine if the extended delay in flowering of the \(\text{vrn1-} \) null mutants was mediated by the flowering repressor \(\text{VRN2} \), we compared heading times of the unvernalized sib lines \(\text{vrn1-} \text{vrn2-null} \) (set 2) and \(\text{vrn1-} \text{vrn2-null} \) (set 2). These two lines carry the same \(\text{VRN1} \) splice mutations but differ in the presence or absence of functional \(\text{VRN2} \) genes. On average, the unvernalized \(\text{vrn1-} \text{vrn2-null} \) plants headed 63 days earlier than the unvernalized \(\text{vrn1-} \text{vrn2-null} \) sib lines (Figure 2C), which indicates that the \(\text{VRN2} \) gene has a large effect on the late flowering phenotype of the \(\text{VRN1} \) null mutants.

Even though the unvernalized \(\text{vrn1-} \text{vrn2-null} \) flowers 2 months earlier than the \(\text{vrn1-} \) null mutants, they still flowered 37 days later than the unvernalized \(\text{vrn1-} \text{vrn2-null} \) control that carries a functional vernalization-insensitive \(\text{VRN1} \) allele (Table 1). Since these two mutants differ only in the presence or absence of the \(\text{VRN1} \) allele, it can be concluded that this allele has the ability to accelerate flowering even in the absence of \(\text{VRN2} \) (Figure 2C).

A comparison between the unvernalized and vernalized \(\text{vrn1-} \text{vrn2-null} \) plants showed that the vernalized plants flowered on average 23 days earlier than the unvernalized ones (Figure 2C, \(P=0.0001\)). This result indicates that the redundant genes responsible for flowering in the absence of functional \(\text{VRN1} \) proteins are able to respond to vernalization even in the absence of \(\text{VRN2} \).

**Effect of the \(\text{VRN1} \) mutations on transcript levels of \(\text{VRN2} \) and \(\text{FT} \)**

To understand better the large differences in flowering time observed in the \(\text{VRN1} \) and \(\text{VRN2} \) mutants we studied the effect of these mutations on the transcription profiles of \(\text{VRN1} \), \(\text{FT} \) and \(\text{VRN2} \) by quantitative RT-PCR (qRT-PCR). Since \(\text{FT} \) and \(\text{VRN1} \) transcription profiles are similar, they are described together.

**\(\text{FT} \) and \(\text{VRN1} \).** In both unvernalized and vernalized plants, \(\text{VRN1} \) (Figure 3A–3B) and \(\text{FT} \) (Figure 3C–3D) transcript levels were up-regulated earlier during development in the spring \(\text{vrn1-} \) \text{vrn2-null} \) mutants (blue lines) than in the other genotypes, in agreement with their early heading time. No significant differences in the expression profiles of these genes between Kronos and the \(\text{vrn1-} \text{vrn2-null} \) mutants were detected, which is consistent with the very similar heading times of these lines (data not shown). The winter \(\text{vrn1-} \text{vrn2-null} \) mutants (red lines) showed a significant up-regulation of \(\text{VRN1} \) during vernalization (Figure 3B) as expected for a line carrying a functional vernalization-responsive \(\text{VRN1} \) allele.

In the \(\text{vrn1-} \text{vrn2-null} \) mutants, transcript levels of the mutant \(\text{VRN1} \) alleles increased significantly by the end of vernalization (\(P=0.01\)). However, since it is not possible to separate the effect of the truncated \(\text{VRN1} \) proteins on its transcriptional regulation from the effects of the mutations on RNA stability, the \(\text{VRN1} \) transcript profiles in the \(\text{vrn1-} \text{vrn2-null} \) mutants were excluded from the figures and were not discussed further. The \(\text{vrn1-} \text{vrn2-null} \) mutants took a long time to flower, but at heading time the transcript levels of \(\text{FT} \) in the flag leaves were high in both vernalized and unvernalized plants (Figure 3, dotted lines). Almost identical transcription profiles were observed in the second set of \(\text{VRN1} \) mutants (Figure S2A–S2D) confirming the previous results.

**\(\text{VRN2} \).** In the unvernalized plants, an inverse correlation was observed between the transcript levels of \(\text{VRN1} \) and \(\text{VRN2} \), similar to the one described in previous studies [22]. The \(\text{vrn1-} \) \text{vrn2-null} \) mutants (Figure 3E, blue solid lines) showed the highest transcript levels of \(\text{VRN1} \) and the lowest transcript levels of \(\text{VRN2} \) and the opposite was observed in the \(\text{vrn1-} \) \text{vrn2-null} \) winter mutants (Figure 3E, red and green solid lines).

The comparison of the \(\text{VRN2} \) transcript profiles among the different genotypes revealed two important results. First, the \(\text{VRN2} \) genes were down-regulated during vernalization in both the \(\text{vrn1-} \) \text{vrn2-null} \) and \(\text{vrn1-} \text{vrn2-null} \) mutants (Figure 3F, \(P=0.0002\), and Figure S2F, \(P=0.0009\)). This result shows for the first time that \(\text{VRN1} \) is not essential for the down-regulation of \(\text{VRN2} \) by vernalization. Second, a rapid increase in the transcript levels of \(\text{VRN2} \) to pre-vernization levels was observed two weeks after the vernalized plants were returned to room temperature in the \(\text{vrn1-} \) \text{vrn2-null} \) mutants but not in the other genotypes (Figure 3F, RT). At the same time point, the \(\text{vrn1-} \text{vrn2-null} \) mutants showed lower transcript levels of \(\text{FT} \) (Figure 3D, RT) and \(\text{VRN1} \) (Figure 3B, RT) than the

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**Table 1. Description of \(\text{VRN1} \) and \(\text{VRN2} \) mutants.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vernalization</th>
<th>(\text{VRN1} ) (flowering promoter)</th>
<th>(\text{VRN2} ) (flowering repressor)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Functional</td>
<td>Non-functional</td>
</tr>
<tr>
<td><strong>Spring growth habit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (wt)</td>
<td>Insensitive</td>
<td>(\text{VRN-A1} ) (\text{VRN-B1} )</td>
<td>None</td>
</tr>
<tr>
<td>(\text{vrn1-} )</td>
<td>Insensitive</td>
<td>(\text{VRN-A1} )</td>
<td>(\text{vrn-B1} )</td>
</tr>
<tr>
<td>(\text{vrn1-} \text{vrn2-null} )</td>
<td>Insensitive</td>
<td>(\text{VRN-A1} )</td>
<td>(\text{vrn-B1} )</td>
</tr>
<tr>
<td><strong>Winter growth habit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{vrn1-} )</td>
<td>Sensitive</td>
<td>(\text{VRN-B1} )</td>
<td>(\text{vrn-A1} )</td>
</tr>
<tr>
<td>(\text{vrn1-} \text{vrn2-null} )</td>
<td>Sensitive</td>
<td>None</td>
<td>(\text{vrn-A1} ) (\text{vrn-B1} )</td>
</tr>
</tbody>
</table>

1. Vernalization accelerates flowering in the vernalization sensitive genotypes but has no effect in the vernalization insensitive ones.
2. The Kronos line used for mutagenesis carries a vernalization insensitive \(\text{VRN-A1} \) allele with a large deletion in the first intron and a vernalization responsive \(\text{VRN-B1} \) allele (wild type). The presence of the vernalization insensitive \(\text{VRN-A1} \) allele results in a spring growth habit independently of the allele present at the \(\text{VRN-B1} \) locus [7].

doi:10.1371/journal.pgen.10003134.t001

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PLOS Genetics | www.plosgenetics.org 4 December 2012 | Volume 8 | Issue 12 | e1003134

*Wheat VERNALIZATION1 Knock-Out Mutants*
Δvrn-A1 mutants. Similar results were confirmed in the second set of Δvrn-1-null mutants (Figure S2F).

We conclude from these results that an important role of VRN1 in the leaves is to maintain the repression of the VRN2 genes after vernalization. The inability of the Δvrn-1-null mutants to maintain low levels of VRN2 expression after vernalization correlates well with the reduced vernalization response of the Δvrn-1-null mutants relative to the Δvrn-A1 mutants (Figure 2A).

Even though the Δvrn-1-null mutants showed a large delay in flowering time they eventually flowered. At heading time the transcript levels of VRN2 in the flag leaves were almost undetectable (Figure 3E-3F and Figure S2E-S2F) and the transcript levels of VRN1 and FT were relatively high (Figure 3A-3D and Figure S2A-S2D dotted lines), both in the vernalized and unvernalized plants. These results indicate that VRN2 can be down-regulated during development independently of VRN1.

Effect of the VRN2 mutations on FT transcript levels

To study the role of VRN2 in the absence of functional VRN1 proteins, we compared the transcriptional profiles of the Δvrn-1-null and Δvrn-1-Δvrn-2-null mutants. These two mutants have the same VRN1 mutations but differ in the presence or absence of functional VRN2 genes (Figure 4). Since these two mutants lack any functional VRN1 genes, only the transcript profiles of VRN2 (ZCCT2) and FT are presented in Figure 4.

VRN2 (ZCCT2). The Δwnl-null mutants showed higher levels of ZCCT2 transcripts than the Δwnl-Δwn2-null mutants (Figure 4A). This is expected since the Δwnl-Δwn2-null mutants have a deletion encompassing the ZCCT-B2 gene, and only the transcripts of the non-functional ZCCT-A2 gene are detected in this line. The down-regulation of ZCCT2 during vernalization was similar in both genotypes. However, when plants were returned to room temperature the up-regulation of ZCCT2 was higher in the Δwnl-null than in the Δwnl-Δwn2-null mutants (Figure 4B).

FT. The Δwnl-null mutants (functional VRN2) showed undetectable levels of FT both in the vernalized and the unvernalized plants during this experiment. In contrast, the Δwnl-Δwn2-null mutants (non-functional VRN2) showed very high transcript levels of FT by the end of the experiment, both in the vernalized and unvernalized plants (Figure 4C-4D). The significantly higher levels of FT observed in the Δwnl-Δwn-2-null than in the Δwnl-null mutants correlate well with the two-month flowering difference observed between these two mutants (Figure 2C). These results also demonstrate that the ability of VRN2 to repress FT transcription is not dependent on VRN1.

Interestingly, FT transcript levels in the Δwnl-Δwn2-null mutants jumped from undetectable levels to 18-fold ACTIN two weeks after the plants were removed from vernalization. In the unvernalized plants, FT increased only from 0.1 to 1.8-fold ACTIN during the last two weeks of the experiment. This result is


doi:10.1371/journal.pgen.1003134.g002

PLOS Genetics | www.plosgenetics.org 5 December 2012 | Volume 8 | Issue 12 | e1003134
consistent with the significant acceleration in flowering time observed in the Δvrn1-Δvrn2-null mutants (23 days) after eight weeks of vernalization.

Preliminary characterization of VRN1’s closest paralogs FUL2 and FUL3

Even in the absence of functional VRN1 proteins, vernalization accelerated flowering of the Δvrn1-null and Δvrn1-Δvrn2-null mutants (Figure 2C), which indicates the existence of additional vernalization responsive genes. To test if VRN1’s closest paralogs FUL2 and FUL3 were responsive to vernalization, we characterized their expression profiles using the same cDNA samples obtained from the vernalization experiments described in Figure 3.

FUL2 and FUL3 are expressed at high levels in the leaves. In the leaves of unvernalized VRN1 mutants, FUL2 and FUL3 transcripts levels increased during development reaching high levels in the flag leaves (>6-fold ACTIN, Figure S3A and S3C), that were even higher than those described previously for VRN1 (Figure 3). Similar to VRN1 (Figure 3), the up-regulation of FUL2 and FUL3 transcripts levels occurred earlier in development in the spring Δvrn-B1 mutants than in the winter Δvrn-A1 and Δvrn1-null mutants (Figure S3).

FUL2 and FUL3 transcript levels are up-regulated by vernalization independently of VRN1. FUL2 and FUL3 transcript levels were significantly (P<0.01) up-regulated by vernalization in the leaves of the Δvrn1-null mutants, both in set 1 (Figure 5A–5D) and set 2 (Figure S4A–S4B). After six weeks of vernalization, FUL3 transcript levels in the Δvrn1-null mutants reached levels similar to those observed for VRN1 (10–15% ACTIN level), but FUL2 transcript levels were more than 20-fold lower than VRN1

Figure 3. qRT–PCR transcriptional profiles of VRN1, FT, and ZCCT2 (=VRN2) in mutant set 1 (premature stop codon). A–B) VRN1, C–D) FT, E–F) ZCCT2. Left panels A, C and E) unvernalized plants. Right panels B, D, and F) vernalized plants. Δvrn-A1: mutation in VRN-A1 (winter, functional vernalization responsive VRN-B1 allele), Δvrn-B1: mutation in VRN-B1 (spring, functional vernalization insensitive VRN-A1 allele), Δvrn1-null: truncated VRN1 proteins. Blue shaded areas indicate vernalization at 4°C under long days. 0 wV: 3 weeks-old plants grown under 22°C/17°C (day/night) conditions before vernalization, 3 wV: 3 weeks of vernalization, 6 wV: 6 weeks of vernalization, RT: two weeks after returning the vernalized plants to pre-vernalization conditions. A final sample was obtained from flag leaves at heading times (FL, dotted lines), which are indicated in days from sowing to heading. The X axis scale is not proportional to time and the Y scale is in fold-ACTIN values. Error bars are SE of the means from 8 biological replications.

doi:10.1371/journal.pgen.1003134.g003
Figure 4. Comparison of *VRN2* and *FT* transcript levels between Δ*vrn1-null* and Δ*vrn1-Δvrn2-null mutants. A–B) ZCCT2, C–D) FT. Left panels A and C) unvernalized plants. Right panels B and D) vernalized plants. Δ*vrn1-null*: no functional VRN1 proteins (functional VRN2, winter), Δ*vrn1-Δvrn2-null*: truncated VRN1 and VRN2 proteins (early). Blue shaded areas indicate vernalization at 4°C under long days. 0 wV: 3 weeks-old plants grown under 22°C/17°C (day/night) conditions before vernalization, 4 wV: 4 weeks of vernalization, 8 wV: 8 weeks of vernalization, RT: two weeks after returning the vernalized plants to pre-vernalization conditions. A–B) ZCCT2 (Δ*VRN2*). Lower transcript levels in the Δ*vrn1-Δvrn2-null* mutant (red line) are likely caused by the complete deletion of the *VRN-B2* genes in this line. Only the non-functional *ZCCT-A2* transcripts are detected, C–D = *FT*. Note the rapid up-regulation of *FT* in the Δ*vrn1-Δvrn2-null* mutants relative to the Δ*vrn1-null* mutants. The X axis scale is in weeks (w) and is not proportional to time. The Y scale is in fold-TECT values. Error bars are SE of the means from 8 biological replications.

doi:10.1371/journal.pgen.1003134.g004

(FUL2 and FUL3 are positively regulated by FT.) The elimination of all functional copies of *VRN2* in the Δ*vrn1-Δvrn2-null* mutants resulted in a significant up-regulation of *FT* in the leaves after vernalization relative to the Δ*vrn1-null* mutants (Figure 4D), which suggested the possibility that the negative effect of *VRN2* on *FUL2* and *FUL3* was mediated by *FT*. To test this hypothesis, we analysed the transcript levels of *FUL2* and *FUL3* in two pairs of isogenic lines differing in *FT* transcript levels and heading time [5] (Figure S6A).

The first pair of isogenic lines included the late-spring variety Chinese Spring (CS) and a substitution line of chromosome 7B from the variety Hope in CS (henceforth, CS-H7B). The Hope 7B chromosome carries an *FT* allele with an insertion of a repetitive element in its promoter that is associated with high transcript levels of *FT* [5]. The second pair of isogenic lines included the winter wheat variety Jagger (JAG) and transgenic Jagger plants (JAG-OE). The transgenic wheat variety Jagger (JAG) and transgenic Jagger plants (JAG-OE) transformed with the *FT* allele from Hope [5]. The transgenic JAG-OE and the chromosome substitution line CS-H7B flowered significantly earlier (*P* < 0.0001) than their respective controls (Figure S6A).

After 7 weeks at room temperature, *FT* transcript levels remained undetectable in the lines carrying the wild type *FT* alleles (CS and JAG), but were 7 and 21-fold higher than *ACTIN* in CS-H7B and JAG-OE, respectively (Figure S6B). The higher levels of *FT* in CS-H7B and JAG-OE were associated with

(FUL2 and FUL3 are negatively regulated by VRN2.) In contrasts with the Δ*vrn1-null* mutants, the transcript levels of *FUL2* and *FUL3* in the leaves of the Δ*vrn1-Δvrn2-null* mutants, increased to very high levels (>10-fold *ACTIN*, Figure S5A–S5B) when the vernalized plants were returned to room temperature. Since these two sister mutant lines differ only in the presence of functional *VRN2* genes, these results indicate that *VRN2* has a negative effect on the transcriptional regulation of *FUL2* and *FUL3* in the leaves. As described below, this effect is likely mediated by the negative effect of *VRN2* on *FT* transcription.

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After 7 weeks at room temperature, *FT* transcript levels remained undetectable in the lines carrying the wild type *FT* alleles (CS and JAG), but were 7 and 21-fold higher than *ACTIN* in CS-H7B and JAG-OE, respectively (Figure S6B). The higher levels of *FT* in CS-H7B and JAG-OE were associated with

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siGnificantly higher transcript levels of FUL2 and FUL3 relative to the control lines (Figure S6B and S6C), which suggests that these two genes are positively regulated by FT.

Discussion

**VRN1 is not essential for wheat flowering**

The dramatic non-flowering phenotype of the wheat *mp* mutants suggested initially that VRN1 was an essential flowering gene [33]. However, a later study showed that the deletions in the *mp* mutants including VRN1 were larger than initially proposed, and encompassed several genes including PHYC, an important light receptor and AGLG1, the wheat ortholog of rice PAP2 [14]. Phytochromes affect flowering time in Arabidopsis [35] and rice [36] and PAP2 affects flowering time and reproductive development in rice [32] and therefore cannot be ruled out as an alternative cause of the non-flowering phenotype of the *mp* mutants.

The Δvrn1-null mutants developed in this study allowed us to separate the effect of VRN1 from the effect of the other genes included in the *mp* deletions. The production of normal flowers and seeds in the Δvrn1-null and Δvrn1-vm2-null mutants demonstrates that VRN1 is not essential for wheat flowering, and that the non-flowering phenotype of the *mp* mutants is not solely determined by the deletion of VRN1. In addition, the early flowering time of the Δvrn1-vm2-null mutant indicates the existence of redundant flowering genes that are capable of rapidly inducing flowering in wheat in the absence of functional VRN1 and VRN2 proteins.

Even in the absence of VRN1 there is a significant vernalization response

Vernalization accelerated flowering time by 84–133 days in the two Δvrn-A1 mutants (functional vernalization responsive VRN-B1 allele), but only by 31–37 days in the Δvrn1-null mutants relative to the unvernalized controls. This three- to four-fold reduction in the acceleration of flowering by vernalization in the mutants with truncated copies of VRN1 confirms the important role this gene plays in the vernalization response in wheat. However, the fact that a significant acceleration of flowering by vernalization was still detected in the Δvrn1-null mutants indicates the existence of unidentified genes with the ability to respond to vernalization. This significant response to vernalization in the absence of VRN1 does not seem to be dependent on the presence of VRN2, because
VRN1 plays a central role in the repression of VRN2 in the leaves after winter

Lukkojanov and collaborators [22] observed a negative correlation between the transcript levels of VRN1 and the transcript levels of VRN2 in the leaves of isogenic hexaploid wheat lines carrying different VRN1 spring alleles, and hypothesized that VRN1 functions as a negative transcriptional regulator of VRN2. This negative interaction became an integral part of a feedback regulatory loop placed at the center of current flowering models (Figure 1), but has never been conclusively demonstrated before this study.

Results from the Δvrn1-null mutants confirmed the validity of the previous hypothesis. Only the mutant lines with no functional copies of VRN1 showed an up-regulation of VRN2 transcript to pre-vernalization levels when the plants were removed from the vernalization treatment and were transferred back to room temperature (Figure 3F and Figure S2F). This result, observed in the two independent sets of Δvrn1-null mutants, confirmed that one specific role of VRN1 is to maintain low transcript levels of VRN2 in the leaves following winter, when the longer day length and warmer weather conditions favour the induction of VRN2 transcription. The transcriptional activation of VRN1 in the leaves during vernalization provides an important regulatory signal that controls the transition from vegetative growth in the fall to reproductive development in the spring. In the fall, low VRN1 transcript levels result in high VRN2 expression, the repression of FT, and the maintenance of vegetative growth. During the winter VRN1 is up-regulated and VRN2 is down-regulated. The presence of VRN1 in the leaves prevents VRN2 from being up-regulated during the longer and warmer days of spring, which facilitates the transcriptional activation of FT and the induction of flowering. In summary, the negative regulation of VRN2 by VRN1 in the leaves is a key regulatory step in the seasonal responses of winter wheat. It remains to be determined whether the VRN1 protein interacts directly with the VRN2 gene or whether other genes mediate this regulatory interaction.

VRN1 is not required for the down-regulation of VRN2 during vernalization and during development of unvernalized plants

Previous vernalization experiments conducted under short-day conditions demonstrated that VRN1 transcript levels were up-regulated by vernalization in the absence of detectable levels of VRN2 and FT transcripts [23,24]. Based on these results it was assumed that vernalization operated mainly on VRN1 and that the downregulation of VRN2 during vernalization was likely an indirect effect of the up-regulation of VRN1 [12,37]. This hypothesis was reinforced by the observation that vernalization promotes an active chromatin state in VRN1 regulatory regions but not in those of VRN2 or FT [25].

However, in this study we observed down-regulation of VRN2 during vernalization in both sets of Δvrn1-null mutants, which encode for truncated VRN1 proteins (Figure 3F and Figure S2F). This result demonstrates that the down-regulation of VRN2 during vernalization does not require the presence of VRN1.

Similarly, a developmental down-regulation of VRN2 was observed in both sets of unvernalized Δvrn1-null plants (Figure 3E and Figure S2E, dotted lines). This result suggests that, in addition to VRN1, there are other negative regulators of VRN2 that are developmentally regulated.

We are currently developing a triple vrn1-null2-null3 mutant to test if the induction of FUL2 and/or FUL3 transcripts during vernalization (Figure 5 and Figure S4A–S4B) and development (Figure S3A and S3C) play a role in the down-regulation of VRN2.

Results from the VRN1 mutants shed light on conflicting flowering models

In rice, the simultaneous down-regulation of closely related FT paralogs Ha2a and RFT1 by RNAi results in non-flowering plants [38]. Therefore, it is tempting to speculate that the permanent down-regulation of FT in the mvp mutants could account for their non-flowering phenotype.

To explain the low levels of FT transcripts detected in the mvp mutants, Shimada and collaborators (2009) proposed an alternative flowering model for the temperate cereals in which VRN1 promotes FT transcription independently of VRN2 [13] (Figure 6A). In this model, the low FT transcript levels of the mvp mutants are caused by the VRN1 deletion. This model also proposes that FT acts as a transcriptional repressor of VRN2, and VRN2 represses VRN1 [13] (Figure 6A). Shimada’s model includes a regulatory feedback loop including the same three genes present in the model in Figure 1 but operating in the opposite direction, and will be referred hereafter as the ‘reverse’ model (Figure 6A).

The simplified model from Figure 1, included for comparison in Figure 6B, will be referred to as the ‘original’ model for the following discussion.

The reverse model assumes that the deletion of VRN1 is the cause of the low transcript levels of FT in the mvp mutants. However, our results indicate that FT can reach very high transcript levels in the absence of functional VRN1 proteins both in the Δvrn1-null and Δvrn1-vrn2-null mutants (Figure 3C–3D, Figure S2C–S2D, Figure S5B). Although, we cannot completely rule out the very unlikely possibility that the truncated VRN1 proteins in the Δvrn1-null mutants are able to up-regulate FT, we favor the hypothesis that the permanent down-regulation of FT in the mvp mutants is caused by the deletion of additional genes closely linked to VRN1 and included in the same deletion. The PHYC is a potential candidate to explain these differences since mutations in the PHYC gene are known to affect flowering time in both rice [36] and Arabidopsis [35]. However, the effects described in these model species are not as large as those observed in the mvp mutants, suggesting the possibility that the non-flowering phenotype of the mvp mutants is caused by other genes in the deletion or by the combination of deletions of more than one gene. We are currently developing a wheat phyC-null mutant to test the contribution of this gene to the regulation of flowering time in wheat. Another potential candidate among the genes deleted in the mvp mutants is the MADS-box gene AGL11. The rice homolog of AGL11 (PAP2) is expressed in the shoot apical meristem during the initiation of the reproductive stage at the same time as the meristem identity genes (OaMADS14 = VRN1, OaMADS15 = FUL2).
and OsMADS18 = FUL3). A null allele of the PAP2 locus combined with reduced expression of the three meristem identity genes by RNA interference down-regulates FT expression in the leaves and inhibits the transition of the apical meristem from the vegetative to reproductive phase [32]. In wheat, AGL61 transcripts are detected in the developing spike later than VRN1 and are not detected in the leaves [1]. These results suggest that these homologous genes play different roles in the regulation of flowering initiation in rice and wheat.

The transcription profiles of FT and VRN2 in the Δvrn1-null and Δvrn1-ful2-null mutants provide additional evidence that contradicts the predictions of the ‘reverse’ flowering model. This comparison shows that even in the absence of functional copies of VRN1, the deletion of VRN2 results in the up-regulation of FT (Figure 4C–4D). This result is difficult to explain by the reverse model, which proposes that the negative regulation of FT by VRN2 requires the presence of functional VRN1 proteins (Figure 6A).

In addition to the genetic evidence discussed above, biochemical interactions described in previous studies support the interactions proposed in the ‘original’ model. The proposed regulation of VRN1 by FT (Figure 6B) in wheat is consistent with the known interactions between FT and AP1 (VRN1 homolog) in Arabidopsis. In this species, the FT protein is synthesized in the leaves and is transported through the phloem to the stem apical meristem where it forms a complex with the bZIP transcription factor FD and binds to the promoter of AP1 [17]. Similarly, the wheat FT protein interacts with the bZIP transcription factor FD12 which interacts in vitro with the VRN1 promoter [18]. In rice, the FT homolog (Hd3a) interacts with OsFD1 and a 14-3-3 protein to form a complex that binds the promoter of the rice homolog of FUL2 (OsMADS15) [39]. A biochemical mechanism has been proposed also for the repression of FT by VRN2 [15]. Using a yeast-three-hybrid system Li et al. (2011) showed that VRN2 and CONSTANS proteins compete for binding with a common set of NF-Y transcription factors, which have been shown to be involved in the regulation of FT in Arabidopsis [20,21]. In summary, available genetic and biochemical evidence support the ‘original’ model of flowering for the temperate cereals [12,27].

Future directions

The fact that both sets of Δvrn1-null null mutants were able to flower and set normal seeds, demonstrated the existence of redundant flowering genes with meristem identity functions overlapping those of VRN1. The MADS-box genes FUL2 and FUL3 are the closest paralogs of VRN1 [26] and therefore, the most parsimonious hypothesis is that their encoded proteins have retained meristem identity functions that can compensate for the lack of a functional VRN1 protein. A similar redundant system exists in Arabidopsis, in which only simultaneous mutations in the three duplicated meristem identity genes API–CAL–FUL results in mutants that are unable to form flowers under all tested environmental conditions [31]. The existence of functional redundancy in the meristem identity genes is also evident in rice, where the down-regulation of individual meristem identity genes OsMADS14 (VRN1), OsMADS15 (FUL2) or OsMADS18 (FUL3) has limited effect on flowering time [32,40]. Only the simultaneous downregulation of the three rice meristem identity genes in combination with pap2 mutants resulted in extended delays in stem elongation and severely perturbed inflorescence development [32].

In wheat and barley, previous studies have provided some indirect evidence that support the hypothesis that FUL2 and FUL3 have retained overlapping functions with VRN1. First, in situ hybridization studies have shown similar spatial and temporal transcription profiles of these three genes during the initial stages of spike development [29,30,41,42]. In addition, wheat FUL2 and rice FUL3 genes were shown to induce flowering when over expressed in transgenic Arabidopsis and rice plants [30,40].

The experiments described in this study showed additional similarities between FUL2, FUL3 and VRN1 genes. First, the transcript levels of FUL2 and FUL3 were significantly up-regulated during vernalization independently of VRN1 and this up-regulation was proportional to the duration of the cold treatment, as described before for VRN1 (Figure 5 and Figure S4A–S4B). Second, the transcript levels of FUL2 and FUL3 were negatively regulated by VRN2 (Figure 5 versus Figure S5A–S5B) and positively regulated by FT in transgenic wheat plants overexpressing FT (Figure S6B–S6D). Finally, when Δvrn1-null mutant plants were moved from the cold to room temperature, FUL2 and FUL3 transcripts returned to pre-vernalization levels in the mature leaves but not in the actively dividing apices (Figure 5). Since cell division is required to establish epigenetic changes, the different transcriptional profiles in these two tissues may reflect the epigenetic regulation of FUL2 and FUL3 by vernalization, as shown before for VRN1 [25]. Studies of the chromatin changes in the regulatory regions of FUL2 and FUL3 will be required to rule out alternative explanations. In summary, the similar patterns of transcriptional regulation, together with the similar in situ hybridization profiles in early spike development and the early flowering of FUL2 and FUL3 transgenic plants, support the existence of some overlapping functions between FUL2, FUL3 and VRN1. We have initiated the development of a triple vrn1–ful2–ful3 mutant in tetraploid wheat to test the roles of FUL2 and FUL3 in flowering initiation in wheat.

In addition to the above similarities, a critical difference was observed in the ability of these three meristem identity genes to regulate VRN2 expression in the leaves. In the absence of functional VRN1 genes, VRN2 was up-regulated after vernalization in the Δvrn1-null mutants, in spite of the presence of functional FUL2 and FUL3 genes. This result suggests that FUL2 and FUL3 are unable to maintain the repression of VRN2 after vernalization.

In summary, this study demonstrates that a central role of VRN1 in the leaves is to maintain the repression of VRN2 after the winter to promote timely flowering in the spring. In spite of its important role in the seasonal regulation of flowering, our results demonstrate that VRN1 is not essential for flowering and raise new questions regarding the roles of FUL2, FUL3, AGL61 and PHIC in the regulation of flowering initiation in wheat. Flowering time is a key component of wheat adaptation and productivity and therefore, a precise understanding of the regulatory mechanisms involved in the initiation of flowering will be beneficial to engineer wheat cultivars better adapted to a changing environment.

Materials and Methods

Mutant screen

A TILLING (for Targeting Local Lesions IN Genomes) population of 1,368 lines of the tetraploid wheat cultivar ‘Kronos’ mutagenized with ethyl methane sulphonate (EMS) [34] was screened for mutations in the A and B genome copies of VRN1 using the genome specific primers described in Table S1. Two VRN1 genomic regions, one including exon one and the other including exons three to six, were targeted for mutant detection using the Cell assay described before [34]. The VRN2 natural mutants have been described before [14].

qRT–PCR

RNA samples were extracted from leaves using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). Purified RNA samples were
checked for RNA integrity by running 0.5 μg RNA on a 1% agarose gel. All samples showed clear 18S and 25S ribosomal RNA bands indicating lack of RNA degradation. Melting curves showed a single peak, which confirmed amplification of a single product. Standard curves were constructed to calculate amplification efficiency for the SYBR Green systems developed for FUL2 and FUL3 (Table S3). SYBR Green systems for VRN1, ZCCT2 and FT were developed in previous studies [5,11]. All primers used for qRT-PCR are conserved between the A- and B-genome copies of their respective targets. Quantitative PCR was performed using SYBR Green and a 7500 Fast Real-Time PCR system (Applied Biosystems). ACTIN was used as an endogenous control using primers described before [43].

Transcript levels for all genes and experiments presented in this study are expressed as linearized fold-

\[ \frac{\text{ACTIN}}{\text{TARGET}} \]

The resulting number indicates the ratio between the initial number of molecules of the target gene and the number of molecules of ACTIN and therefore, the Y scales are comparable across genes and experiments. Primer efficiencies were all higher than 95% (Table S3) and therefore, were not included in the calculation of the linearized values. In the Δavl1-null set 2 and the derived Δavl1-vrn2-null mutants the VRN-B1 transcripts were not detected in the qRT-PCR experiments.

Experimental conditions for the different experiments

The flowering experiments for the VRN1 mutants were performed in CONVIRON growth chambers. Long day photoperiod experiments were performed with 16 h of light (6 am to 10 pm, including 1 hour ramp at the beginning and end of the cycle) and 8 h of dark. Intensity of the sodium halide lights measured at plant head height was 260 μm s⁻¹. For the unvernalized plants day temperatures were set at 22°C and night temperatures at 17°C respectively. Relative humidity in the chambers was maintained at 60% throughout the entire experiment.

All the vernalization experiments in this study were performed under long days to separate the effect of low temperature from the overlapping effects of short days. Day and night temperatures were 4°C whereas light intensity and relative humidity were identical to the unvernalized experiments. Unvernalized control plants were planted 5 weeks after the sowing of the vernalized plants to allow both groups of plants to reach a similar number of leaves by the end of the vernalization treatment. Even though the developmental stages of the two groups were coordinated, the vernalization treatment can still affect the subsequent growth rates after vernalization. To eliminate this potential difference, spring lines with no vernalization requirement were included as controls in both vernalized and unvernalized treatments. The differences in heading time between the vernalized and unvernalized vernalization-insensitive spring lines were used to adjust the heading times of the other vernalized plants. The Δavl-B1 mutants were used as controls in Figure 2A and the Δavl-B1-Δavl2-null mutant was used as a control in Figure 2C. Both control lines carry the functional vernalization-insensitive VRN-A1 allele. The Δavl-B1-Δavl2-null mutant has the additional deletion of all functional copies of VRN2 and differs from the Δavl1-Δavl2-null lines in the presence of the functional VRN-A1 allele.

The experiment comparing the Δavl1-null and Δavl1-vrn2-null mutants (Figure 2C) was performed in the greenhouse (both mutants were grown under the same conditions); with an average day time and night time temperatures of 25°C and 17°C, respectively. Day length was extended to 16 h with high pressure sodium lights. Tissues for RNA extraction and qRT-PCR were collected at the same time (10:30 am) in all experiments to avoid potential confounding effects of circadian rhythms. Vernalization in this experiment was performed as described above.

To study gene expression in the apices (Figure 5B and 5D), we collected apices from Δavl1-null mutants set 1 grown under long days in the greenhouse. Samples were collected before vernalization, six weeks after vernalization (4°C) and two weeks after the plants were moved from the cold to room temperature. At each sampling point, 90 apices were harvested and pooled into three replicates including 30 apices per replicate.

Accession numbers

GenBank accession numbers are JX020745 to JX020760.

Supporting Information

Figure S1 Positions and effects of the different mutations in the selected VRN1 mutants. The G864A mutation in the Δavr-B1Δavr2 splice site mutant not only eliminated the splice site but also introduced a stop codon (TGA) at the same position. A) The black rectangles indicate VRN1 exons. B) Chromatograms of wild type and homozygous mutant alleles. Note that mutations appear as single peaks, which indicates the presence of a single copy of each of the mutated genes.

(TIF)

Figure S2 qRT-PCR transcriptional profiles in the leaves of mutant set 2 (splice site Δavl-A1Δavl1Δavr-B1Δavr2). A-B) VRN1, C-D) FT, E-F) ZCCT2. Left panels A, C and E) unvernalized plants. Right panels B, D, and F) vernalized plants. Blue shaded area indicates vernalization at 4°C under long days. Δavl-B1 mutants are indicated by blue lines (functional Vrn-A1 allele, spring growth habit); Δavl-A1 in red (functional vrn-B1 allele, winter growth habit), and Δavl1-null in green. 0 wV: 3 weeks-old plants grown at 22°C/17°C (day/night) before vernalization, 3 wV: 3 weeks of vernalization, 6 wV: 6 weeks of vernalization, RT: two weeks after returning the vernalized plants to pre-vernalization conditions. A final sample was obtained from the flag leaves (FL) at heading time, which are indicated in days from sowing to heading (adjusted as indicated in Material and Methods in the vernalized plants). The X axis scale is not proportional to time and the Y scale is in fold-ACTIN values (number of molecules of target gene/number of molecules of ACTIN). Error bars are SE of the means from 8 biological replications.

(TIF)

Figure S3 qRT transcriptional profiles of FUL2 and FUL3 in the leaves. A) FUL2, B) FUL3. Left panels A and C) vernalized. Right panels B and D) unvernalized. The blue shaded area indicates vernalization at 4°C under long days. Δavl-B1 mutants are indicated in blue (functional Vrn-A1, spring growth habit), Δavl-A1 mutants in red (avl-B1, winter growth habit), and Δavl1-null mutants in green. 0 wV: 3 weeks-old plants grown at 22°C day/17°C night before vernalization, 3 wV: 3 weeks of vernalization, 6 wV: 6 weeks of vernalization, RT: two weeks after returning the vernalized plants to pre-vernalization conditions. A final sample was obtained from the flag leaves (FL) at heading time, which are indicated in days from sowing to heading (adjusted as indicated in Material and Methods). The X axis scale is not proportional to time and the Y scale is in fold-ACTIN values. Error bars are SE of the means from 8 biological replications. The response of FUL2 and FUL3 to vernalization in the Δavl1-null mutants is shown in a more detail scale in Figure S4.

(TIF)

Figure S4 Transcriptional profiles of FUL2, FUL3 and FT during and after vernalization in the leaves of Δavl1-null mutants
set 2 (spice site mutants). A) FUL3, B) FUL2 and FT. The blue shaded area indicates vernalization at 4°C under long days. 0 wV: 3 weeks-old plants grown at 22°C/17°C (day/night) immediately before vernalization, 3 wV: 3 weeks of vernalization, 6 wV: 6 weeks of vernalization, RT: two weeks after removing the plants from the cold and returning them to pre-vernalization conditions. The X axis scale is not proportional to time and the Y scale is in fold-FTV values. Error bars are SE of the means from 3 biological replications. Note the down-regulation of FUL2, and FUL3 when plants were returned to room temperature, at the same time that the ZCCT2 gene is up-regulated in the Δavr1-null mutants (Figure S2F).

**Figure S5** Transcriptional profiles of FUL2, FUL3 and FT during and after vernalization in the leaves of Δavr1-Δavr2-null mutant (no functional copies of VRN1 or VRN2). A) FUL3, B) FUL2 and FT. The blue shaded areas indicate vernalization at 4°C under long days. 0 wV: 3 weeks-old plants grown at 22°C/17°C (day/night) immediately before vernalization, 4 wV: 4 weeks of vernalization, 8 wV: 8 weeks of vernalization, RT: two weeks after removing the plants from the cold and returning them to pre-vernalization conditions. The X axis scale is not proportional to time and the Y scale is in fold-FTV values. Error bars are SE of the means from 3 biological replications. Compare the strong up-regulation of FUL2, and FUL3 (>9-fold ACTIN) in the Δavr1-Δavr2-null mutant after vernalization (RT) with the down-regulation observed at the same time point in the Δavr1-null mutants (functional VRN2 gene) in Figure S4A and B.

**Figure S6** Heading time and transcription profiles of isogenic lines of hexaploid wheat differing in FT expression levels. A) Heading time of plants grown under long days (16 h light/8 h dark). B-D) qRT-PCR transcription profiles in the leaves. The X axis scale is in weeks (w) and is not proportional to time. The Y scale is in fold-AC15T values. B) FT, C) FUL2 and D) FUL3. Abbreviations: JAG-OE = transgenic Jagger plants transformed with the Hope over-expressing FT allele [5], JAG = control winter wheat cultivar Jagger, CS-H7B = Hope 7B chromosome substitution carrying an over-expressing FT allele in CS, CS = control spring wheat cultivar Chinese Spring. Error bars are SE of the means from 8 biological replications. *** = P<0.0001.

**Table S1** Primers and PCR conditions used for screening the TILLING population and for mutant detection.

**Table S2** Mutations resulting in truncations (spice sites and premature stop codon mutations) and amino acid changes with their respective Position-Specific Scoring Matrix (PSSM) and SIFT scores.

**Table S3** SYBR GREEN quantitative PCR primers for FUL2 and FUL3 and their respective amplification efficiencies.

**Acknowledgments**
We thank Nestor Kipps and Huiqiong Lin for their technical help and Chengxia Li, Stephen Pearce, Gabriela Tranquilli, and Iago Hale for their valuable suggestions.

**Author Contributions**
Conceived and designed the experiments: AG JD. Performed the experiments: AG. Analyzed the data: AG JD. Contributed reagents/materials/analysis tools: AC JD. Wrote the paper: AC JD. Wrote the first and final version of the manuscript: JD.

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