Plant Physiology Preview. Published on April 29, 2010, as DOI:10.1104/pp.109.152488

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Journal Research Area: Development and Hormone Action

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Title: *ODDSOC2* is a MADS box floral repressor that is down-regulated by vernalization in temperate cereals

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Abstract

In temperate cereals, such as wheat and barley, the transition to reproductive development can be accelerated by prolonged exposure to cold (vernalization). We examined the role of the grass-specific MADS box gene *ODDSOC2 (OS2)* in the vernalization response in cereals. The barley (*Hordeum vulgare*) *OS2* gene (*HvOS2)* is expressed in leaves and shoot apices but is repressed by vernalization. Vernalization represses *OS2* independently of *VERNALIZATION1* (*VRN1*) in a *VRN1* deletion mutant of einkorn wheat (*Triticum monococcum*), but *VRN1* is required to maintain down-regulation of *OS2* in vernalized plants. Furthermore, barleys that carry active alleles of the *VRN1* gene (*HvVRN1)* have reduced expression of *HvOS2*, suggesting that *HvVRN1* down-regulates *HvOS2* during development. Over-expression of *HvOS2* delayed flowering and reduced spike, stem and leaf length in transgenic barley plants. Plants over-expressing *HvOS2* showed reduced expression of barley homologues of the Arabidopsis (*Arabidopsis thaliana)* gene *FLORAL PROMOTING FACTOR 1 (FPF1)* and increased expression of *RNase-S-like* genes. *FPF1* promotes floral development and enhances cell elongation, so down-regulation of *FPF1-like* genes might explain the phenotypes of *HvOS2* over-expression lines. We present an extended model of the genetic pathways controlling vernalization-induced flowering in cereals, which describes the regulatory relationships between *VRN1*, *OS2* and *FPF1-like* genes. Overall these findings highlight differences and similarities between the vernalization responses of temperate cereals and the model plant Arabidopsis.

Introduction

Many plants from temperate climates require prolonged exposure to low temperatures to become competent to flower; a phenomenon known as vernalization. The requirement for vernalization is often combined with daylength sensitivity. For example, many ecotypes of Arabidopsis (*Arabidopsis thaliana)* are vernalized during winter, and then flower as daylength increases during spring (Imaizumi and Kay, 2006; Jaeger et al., 2006; Zeevaart, 2006; Turck et al., 2008). Similar seasonal flowering responses are found in economically important cereal crop species including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Trevaskis et al., 2007a; Distelfeld et al., 2009; Greenup et al., 2009).

In Arabidopsis, the promotion of flowering by increasing daylength is mediated by *FLOWERING LOCUS T (FT)* (Kardailsky et al., 1999; Kobayashi et al., 1999). *FT* encodes a mobile florigen which is produced in the leaves in long days and travels to the shoot apex where it promotes floral development (Corbesier et al., 2007). Long-day induction of *FT* in the leaves is controlled by the CONSTANS (CO) protein (Onouchi et al., 2000; An et al., 2004). Expression of the *CO* transcript follows a diurnal rhythm, peaking in the late afternoon (Valverde et al., 2004; Jang et al., 2008). In long-days the peak in *CO* expression occurs in light, which stabilises the CO protein, allowing activation of *FT* (Valverde et al., 2004; Jang et al., 2008).

Winter annual ecotypes of Arabidopsis do not flower rapidly in long days unless plants have been vernalized. This requirement for vernalization is mediated by the MADS box floral repressor *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Sheldon et al., 1999), which represses *FT* and a second floral promoter *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* (Michaels and Amasino, 1999; Sheldon et al., 1999; Hepworth et al., 2002; Michaels et al., 2005). Vernalization down-regulates *FLC*, allowing long-day induction of *FT* and *SOC1* to accelerate flowering. Vernalization-induced repression of *FLC* is mediated by protein complexes that chemically modify histones at the *FLC* locus to promote an inactive chromatin state (Schubert et al., 2006; Wood et al., 2006; De Lucia et al., 2008). The repressive histone modifications deposited at *FLC* chromatin during vernalization are stable, so repression of *FLC* is maintained post-vernalization; this allows long-day induction of *FT* to occur in spring in vernalized plants (Sheldon et al., 2000).

The molecular mechanisms that promote flowering in response to long days in Arabidopsis are conserved in temperate cereals. For instance, *CO* and *FT-like* genes have been identified in barley and related grasses (Turner et al., 2005; King et al., 2006; Yan et al., 2006; Faure et al., 2007). The barley *FT-like 1* gene (*FT1* or *VRN3*) is induced by long days and appears to be the functional equivalent of *FT* in cereals (Turner et al., 2005; King et al., 2006; Yan et al., 2006; Faure et al., 2007). As is the case for *FT* in vernalization-requiring Arabidopsis ecotypes, vernalization is a pre-requisite for long-day induction of *FT1* in vernalization responsive barleys (Hemming et al., 2008). No homologues of *FLC* have been identified in cereals. Instead, *VERNALIZATION2* (*VRN2*) is expressed in long-days to suppress induction of *FT1* and delay flowering prior to vernalization (Takahashi and Yasuda, 1971; Yan et al., 2004; Trevaskis et al., 2006). Vernalization induces expression of *VERNALIZATION1* (*VRN1*) (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; von Zitzewitz et al., 2005), which down-regulates *VRN2* and promotes expression of *FT1* in long days (Trevaskis et al., 2006; Yan et al., 2006; Hemming et al., 2008; Sasani et al., 2009). *VRN1* also promotes inflorescence (spike) initiation at the shoot apex, irrespective of daylength (Trevaskis et al., 2006; Hemming et al., 2008; Sasani et al., 2009). Like *FLC*, changes in chromatin state at the *VRN1* locus might provide a mechanism for a memory of vernalization in cereals by allowing stable activation of *VRN1* by vernalization (Oliver et al., 2009).

The vernalization response has probably evolved independently in Arabidopsis and the temperate cereals (grasses). Here we examine the function of a grass-specific MADS box gene previously identified by gene expression analyses as a potential component of the vernalization response in cereals (*Triticum aestivum MADS box gene 23 - TaMx23,* Trevaskis et al., 2003; *Triticum aestivum AGAMOUS-like 33 - TaAGL33*, Winfield et al., 2009). We show that this gene represses flowering and cell elongation by down-regulating a group of genes related to the *FLOWERING PROMOTER FACTOR1 (FPF1)* gene of Arabidopsis.

Results

ODDSOC2 is a truncated MADS box gene found in cereals and related grasses.

Two barley homologues of *Triticum aestivum MADS box gene 23 (TaMx23)* (Trevaskis et al., 2003) were identified amongst barley ESTs deposited in the GENBANK database. These genes have no direct equivalent in Arabidopsis, but show

weak similarity to *SUPPRESSION OF OVEREXPRESSION OF CONSTANS1 (SOC1)* (Supplemental Table S1, Supplemental Fig. S1). These genes were designated *ODDSOC1* (*HvOS1*) and *ODDSOC2* (*HvOS2*). *ODDSOC-like* genes also occur in a range of cereals other than barley, including wheat, rice, maize and sorghum, and in the model grass *Brachypodium distachyon* (Table I, Supplemental Table S2). All genes share a high degree of sequence identity (Supplemental Fig. S2). A feature common to the predicted ODDSOC-like protein sequences is their short length compared to other plant MADS box proteins (152-167 versus ≥ 200 amino acids). No *ODDSOC-like* genes were identified outside the grasses.

The two *ODDSOC* genes from Brachypodium are closely linked to one another in a region syntenous to barley chromosome 3H (long arm, 134 cM) (Fig. 1A). A single *ODDSOC* gene is found in the rice genome (*OsMADS51,* Kim et al., 2007), located in a syntenous region (Fig. 1A). Phylogenetic analysis showed that *OS-like* genes can be classified into three groups, *OS1* or *OS2* groups in the temperate cereals/grasses plus a third group corresponding to maize, sorghum and rice (Fig. 1B), suggesting these genes have undergone gene duplication during the evolution of the temperate grasses.

HvOS2 **is repressed by vernalization**

HvOS1 and *HvOS2* transcript levels were monitored in seedlings during and after vernalization (Fig. 2A). *HvOS1* transcript levels increased during vernalization, whereas *HvOS2* transcript levels decreased (Fig. 2A). Since down-regulation of the MADS box floral repressor *FLC* plays a central role in the vernalization response of Arabidopsis, the role of *HvOS2* in the vernalization response of barley was examined further. To determine if changes in *HvOS2* expression were maintained after vernalization, transcript levels were assayed in leaves two weeks after plants were removed from the cold. Expression of *HvOS2* remained low in plants that had been vernalized compared to non-vernalized controls (Fig. 2B). *HvOS2* transcript levels were also assayed in the shoot apices, showing that expression of this gene decreases in the apices of vernalized plants (Fig. 2C).

The 5’ UTR of *HvOS2* is not enriched for H3K27 trimethylation

In Arabidopsis histone 3 lysine 27 trimethylation (H3K27me3), a histone modification associated with long-term gene repression, mediates vernalization-induced repression of *FLC* (Bastow et al., 2004; Sung and Amasino, 2004; Finnegan and Dennis, 2007; Schmitz et al., 2008). We examined whether a similar mechanism might mediate vernalization-induced repression of *HvOS2*. H3K27me3 levels were assayed at *HvOS2* before and after vernalization. The level of H3K27me3 near the presumed transcriptional start site at *HvOS2* chromatin was low irrespective of vernalization treatment (Fig. 3A), suggesting that this modification does not play a role in mediating the down-regulation of *HvOS2* during vernalization. The level of H3K4me3, a modification associated with active gene transcription, was lower in vernalized leaves than non-vernalized leaves, consistent with the reduction in *HvOS2* expression following vernalization (Fig. 3B).

Expression of *HvVRN1* is associated with down-regulation of *HvOS2*

HvOS2 expression was compared between 'winter' barleys that respond to vernalization and 'spring' barleys that flower without vernalization. Expression of *HvOS2* was strongest in winter barleys grown without vernalization treatment, and vernalization caused a decrease in *HvOS2* expression in these barleys. Expression of *HvOS2* was low in barleys that flower without vernalization, irrespective of vernalization treatment (Fig. 4A).

To further examine the relationship between vernalization requirement and *HvOS2* expression, *HvOS2* transcript levels were assayed in lines from the Sloop × Halcyon doubled haploid barley population (Read et al., 2003), grown without vernalization. This population segregates for different alleles of *HvVRN1*; a wild-type allele (*VRN1*) which is activated by vernalization and an allele with a deletion in the first intron that is active without vernalization and reduces the vernalization requirement (*VRN1-1*) (Trevaskis et al., 2006, Hemming et al., 2008). Expression of *HvOS2* was lower in lines carrying *VRN1-1* (Fig. 4B), suggesting that *HvVRN1* down-regulates *HvOS2*.

ODDSOC2 expression was then examined in the *MAINTAINED VEGETATIVE PHASE* mutant of the diploid einkorn wheat *Triticum monoccum*, which lacks the *VRN1* gene (Shitsukawa et al., 2007) (hereafter referred to as the *ΔVRN1* mutant). When seedlings were germinated in darkness, without vernalization,

expression of *VRN1* was not detected in either the wild-type parent or the *ΔVRN1* mutant and expression of the *T. monoccum ODDSOC2* gene (*TmOS2*) did not differ (Fig. 4, C and D).

Expression of *TmOS2* and *VRN1* was then examined in seedlings at the end of a seven week vernalization treatment. Expression of *VRN1* was induced in vernalized seedlings of the wild-type parent but was not detected in the *ΔVRN1* mutant (Fig. 4D). Compared to non-vernalized control seedlings, expression of *TmOS2* was lower in vernalized seedlings, irrespective of *VRN1* genotype (WT, P, <0.001 and *ΔVRN1*, P, 0.002) (Fig. 4C). *VRN1* and *TmOS2* transcript levels were then examined in plants that were grown at normal temperatures for 1 or 3 weeks after vernalization treatment. *VRN1* expression remained high in wildtype plants, but was not detected in the *ΔVRN1* mutant (Fig. 4D). In wildtype plants expression of *TmOS2* remained low, but repression of *TmOS2* was not maintained in the *ΔVRN1* mutant (Fig. 4C).

Over-expression of *HvOS2* delays flowering and inhibits leaf and stem elongation

To further investigate the function of *HvOS2*, a spring barley that flowers without vernalization and has low levels of *HvOS2* expression (cv. Golden Promise, see methods and materials) was transformed with a transgene construct that placed *HvOS2* under the control of the maize (*Zea mays*) *UBIQUITIN* promoter. Approximately 50 independent transgenic lines were generated with this construct. The majority were late flowering, compared to non-transformed plants, supporting the hypothesis that *HvOS2* is a repressor of flowering. Two independent transgenic lines, the progeny of which showed segregation for the transgene construct, were characterised in detail; *OxHvOS2-11* and *OxHvOS2-20*. In both these lines expression of *HvOS2* was higher in plants that inherited the transgene (Supplemental Fig. S3) and a late flowering phenotype segregated with the transgene in both transgenic families (Fig. 5A). Plants from the *OxHvOS2-11* line that inherited the transgene flowered on average 14 days later than siblings lacking the transgene (null siblings), which flowered at a similar time to wild type Golden Promise plants. Similarly, *OxHvOS2-20* transgenic plants flowered on average 18 days later than null siblings (Fig. 5A). Comparison of apex morphology at the third leaf stage, the developmental stage when inflorescence initiation typically occurs in Golden Promise plants under these growth

conditions, showed that over-expression of *HvOS2* delays the transition to reproductive development (Fig. 5B).

In addition to influencing flowering time, over-expression of *HvOS2* inhibited leaf elongation. The length of the first and third leaf was reduced in plants over-expressing *HvOS2* (Fig. 5C). This reduction in length was due to decreased cell length; the average length of bulliform cells was significantly reduced in the first and second leaves in the transgenic plants (Fig. 5D and E). The final length of the primary spike was also reduced in transgenic plants over-expressing *HvOS2*, as were primary tiller (stem) internode lengths (Fig. 5F).

The effects of reducing *HvOS2* expression levels was also investigated using gene-specific RNA interference (RNAi) constructs. One of the lines analyzed showed a reduction in expression levels for *HvOS2* (Supplemental Fig. S4A). There were no observable phenotypic abnormalities or any change in heading date/final leaf number in any of the RNAi lines analyzed (Supplemental Fig. S4, B and C).

Over-expression of *HvOS2* down-regulates barley homologues of *Floral Promoting Factor 1*

Microarray analysis of gene expression was used to investigate the molecular basis for the phenotype of plants over-expressing *HvOS2*. RNA from seedlings that over-express *HvOS2* was hybridised to the Affymetrix Barley1 chip (Close et al., 2004) and compared to RNA from null sibling control lines. A total of 90 genes were differentially expressed between plants that over-express *HvOS2* and sibling null controls (2 fold change in expression, $P < 0.05$ Supplemental Table S3). Of the 94 genes differentially expressed, 65 (69%) were up-regulated in plants that over-express *HvOS2* and 25 (27%) were down-regulated. Table II shows the top five up and down-regulated genes that were differently regulated between plants that over-express *HvOS2*.

To verify the results of microarray analysis, the expression levels of several differentially expressed genes were quantified by quantitative RT-PCR (qRT-PCR) (Supplemental Fig. S3). Consistent with the results of microarray analysis, barley orthologs of *Flowering Promoting Factor 1* (*FPF1*), designated *HvFPF1-like1* (Contig HU14G14r) and *HvFPF1-like2* (Contig 18182), were down-regulated in plants over-expressing *HvOS2* (Supplemental Fig. S3, B and C). Conversely, expression levels of two *RNase S-Like* genes, *Hvrsh1* (Gausing, 2000, Contig 5185)

and Hvrsh2 (Contig 5058/9), were elevated in plants over-expressing *HvOS2* (Supplemental Fig. S3, D and E).

FPF1-like genes are regulated by vernalization and daylength in barley

We examined whether expression of *FPF1-like* genes is influenced by vernalization in barley; a response predicted for genes regulated by *HvOS2*. Expression of *HvFPF1-like1* and *HvFPF1-like2* was higher in leaves of vernalized plants compared to non-vernalized plants, when plants were grown in long days post-vernalization (Fig. 6). Vernalization did not influence expression of *FPF1-like* genes when vernalized plants were grown in short days, where expression of *FPF1-like* genes was lower (Fig. 6). These data suggest that, in barley, down-regulation of *HvOS2* by vernalization allows increased expression of *FPF1-like* genes when plants are exposed to long-days.

Discussion

Reproductive development and stem elongation are closely coordinated in temperate cereals. In varieties that require vernalization both processes are delayed until after winter, and begin in spring when temperature and daylength increase. We have identified a MADS box gene from barley, *HvOS2*, which potentially delays the transition to reproductive development and impedes cell elongation in stems and leaves, but is down-regulated by vernalization (Fig. 2). We suggest that *HvOS2* acts in a pathway that delays the transition to reproductive development and inhibits stem elongation prior to winter.

A single *ODDSOC* gene is found in rice; *OsMADS51* (Kim et al., 2007). *OsMADS51* promotes flowering and appears to be a component of the molecular pathway that promotes flowering in response to short-days (Kim et al. 2007). It is possible that *HvOS1* and the equivalent wheat gene *(TaAGL42),* which are induced by vernalization (Fig. 2A) (Winfield et al., 2009), also act as floral promoters. Equally, it is possible that these genes have acquired novel functions after the divergence of rice and the temperate cereals. *HvOS2*, which acts a floral repressor, might have evolved from a duplication of *OsMADS51/ODDSOC1* during the evolution of the vernalization-response pathway in temperate cereals.

HvOS2 is quantitatively down-regulated by cold (longer cold treatments cause stronger down-regulation) and repression of *HvOS2* is maintained in the shoot apex

and leaves after vernalization (Fig. 2B, Supplemental Fig. S5). This pattern of gene expression is similar to *FLC* in Arabidopsis, but unlike *FLC*, repression of *HvOS2* does not appear to involve the deposition of H3K27Me3 (Fig. 3A). Vernalization-induced repression of *TmOS2* does not require *VRN1* (Fig. 4C), but *VRN1* is required to maintain repression of *TmOS2* after vernalization (Figure 4C). *HvVRN1* also down-regulates *HvOS2* in barleys that flower without vernalization (Fig. 4B). Similarly, *VRN1* down-regulates *TmOS2* when *T. monococcum* plants (lacking *VRN2*) are grown in long days (Supplemental Fig. S6). Thus, we suggest that *ODDSOC2* is down-regulated by cold independently of *VRN1*, but *VRN1* represses *ODDSOC2* during development at normal growth temperatures, both following vernalization and in barleys that flower without vernalization. Consistent with this hypothesis, microarray analysis shows that *HvOS2* is repressed by cold treatments that are not long enough to induce *HvVRN1*, but repression is not maintained when plants are returned to normal growth temperatures (Supplemental Fig. S7) (Plexdb, Acc No. BB81, Wise et al., 2008).

Spring barleys that flower without vernalization express *HvOS2* at low levels compared to vernalization-responsive winter barleys (Fig. 4, A and B). Increasing *HvOS2* expression levels in transgenic spring barley (cv. Golden Promise) delayed flowering, suggesting that *HvOS2* functions as a repressor of flowering. The delay of flowering was caused by a delay of the transition to reproductive development, as evidenced by the impact of the *HvOS2* over-expression on final leaf number (Supplemental Fig. S8). Increasing *HvOS2* expression levels also influenced plant growth by inhibiting elongation of cells in the leaves and stems, but did not slow the rate of growth (Supplemental Fig. S8). The phenotypes observed in *HvOS2* over-expression lines are different to those seen when other MADS box genes are over-expressed in barley (see Trevaskis et al. 2007b), suggesting that these phenotypes are indicative of the actual function of *HvOS2* and not simply an artefact of over-expressing a MADS box gene. Further reduction of the already low levels of *HvOS2* expression in Golden Promise by RNA interference did not influence flowering time, however. *HvOS2* activity might be below a functional threshold in this spring barley, which flowers without vernalization. Equally, the level of reduction in *HvOS2* expression by RNAi might not completely eliminate *HvOS2* activity. Isolation of *HvOS2* loss of function mutants could be used to further examine the role of this gene in future studies. Ideally this will be done in a vernalization-responsive cultivar to

allow the functional importance of *HvOS2* to be assessed relative to other genes that delay flowering, such as *HvVRN2*.

The reduction of cell elongation and delay of flowering seen in *HvOS2* over-expression lines was associated with reduced expression of *FPF1*-like genes (Table II and Supplemental Fig. S3, B and C). *FPF1* promotes cell elongation and accelerates flowering when over-expressed in Arabidopsis (Kania et al., 1997; Melzer et al., 1999). These phenotypes mimic the effects of gibberellin (GA) application in Arabidopsis and it has been suggested that *FPF1* acts in a GA-dependent elongation pathway (Kania et al., 1997). Similar phenotypes have also been reported when *FPF1-like* genes were ectopically expressed in rice and tobacco (Ge et al., 2004; Smykal et al., 2004), suggesting that the role of *FPF1-like* genes is conserved across divergent plant lineages. The reduction of cell elongation and delayed flowering seen in *HvOS2* over-expression lines might be due primarily to reduced expression of *FPF1-like* genes. Many of the dwarfing phenotypes in transgenic plants over-expressing *HvOS2* were abolished upon application of GA (Supplemental Fig. S9). This is consistent with the hypothesis that *HvOS2* regulates *HvFPF1-like* genes, which may in turn alter GA responses.

In Arabidopsis expression of *FPF1* increases rapidly at the shoot apex in response to long days (Kania et al., 1997). This long-day response is dependant on *FT* and *CO* (Schmid et al., 2003; Wise et al., 2008) suggesting that *FPF1* acts downstream of *FT* in the long-day flowering-response pathway. The *FPF1-like* genes of barley are also daylength responsive, with elevated expression in long days (Fig. 6), suggesting that this is a conserved feature of *FPF1-like* genes. Expression of *FPF1-like* genes is also determined by vernalization status in barley, the expression of these genes increases in vernalized plants where *HvOS2* expression is reduced. The combined effects of vernalization and long-daylength on *FPF1* gene expression is consistent with a model where down-regulation of *HvOS2* in vernalized plants de-represses *FPF1-like* genes, which are then further induced through a conserved *CO-FT* regulatory pathway as daylength increases during spring (Fig. 7).

Microarray analysis identified other potential targets of *HvOS2*. For example, two *RNase S-like* genes were up-regulated in plants over-expressing *HvOS2* (Table II). These RNases belong to a class that has only been identified in grasses, and predicted to lack RNase activity due to amino acid substitutions at critical residues in the active site (Gausing, 2000). One of these RNases, *rsh1*, is expressed in leaves and

is regulated by light and developmental cues (Gausing, 2000). Expression of both RNase genes decreases during cold treatment, when *HvOS2* is down-regulated (Supplemental Fig. S7) (Plexdb, Acc No. BB81, Wise et al., 2008). This supports the hypothesis that these genes are up-regulated by *HvOS2*. The function(s) of these RNases is not known (Gausing, 2000), so it is unclear whether these genes play a role in vernalized-induced flowering or other biological processes. Analysis of microarray comparisons of gene expression during barley development show that *HvOS2*, the *RNase S-like* and *FPF1-like* genes are all expressed during post-vegetative development (Supplemental Fig. S10, Druka et al., 2008), suggesting broad roles for these genes during plant development in addition to potential roles in regulating flowering time.

In summary, we have identified a novel mechanism by which elongation and flowering are suppressed prior to vernalization in cereals. These findings further highlight the difference between the vernalization pathways of Arabidopsis and cereals, reinforcing the concept that the vernalization response has evolved independently in monocot and dicot plants.

Materials and Methods:

Plant Growth

Barley plants (*Hordeum vulgare*) were grown in glasshouses (18 ±2 ^{o}C) in long days (16-h light/8-h dark), with supplementary light when natural levels dropped below 200µE. For controlled growth conditions plants were grown in growth chambers (20°C) with long (16-h light/8-h dark) or short days (8-h light/16-h dark) under a mix of incandescent and fluorescent lighting. In instances where plants were vernalized, seeds were imbibed and germinated on moist filter paper for 4-7 weeks at 4°C in the dark.

Seeds of the einkorn wheat *(Triticum monoccum) maintained vegetative phase* mutant which lacks *VRN1 (*referred to here as ∆*VRN1)* and the wild-type parent strain were imbibed on moist filter paper. Vernalized samples were grown at 4°C for 7 weeks in the dark and non-vernalized samples were grown in the dark at 22°C to a developmental stage equivalent to that of vernalized seedlings (4cm coleoptile length, vegetative shoot apex). Individual seedlings were ground in liquid nitrogen and a small sample of the ground material was used to extract DNA to determine the genotype of each individual seedling. The remaining material was used to extract

RNA for gene expression studies. Genotyping of individual seedlings was carried out using two sets of primers that annealed to the *TmVRN1* gene (Supplemental Table S4) in a PCR reaction using *Taq* DNA polymerase (New England BioLabs). PCR products were run on a 1.2% Agarose gel and the absence of a visible band was considered an indication of a seedling that was homozygous for the Δ*VRN1* mutation.

Apex dissection and flowering time measurements.

Apices were isolated under a binocular dissecting microscope and then digitally photographed on a Leica M8 digital camera. Leaves were numbered sequentially and plants were grown until the flag leaf emerged to determine Final Leaf Number (FLN). Heading date was measured as the day when the head first emerged from the sheath on the main shoot (Z = 13,21, Zadoks et al., 1974).

Gene Expression Analysis

Total RNA was extracted using the method of Chang et al. (1993) or the Qiagen RNeasy Plant Miniprep kit (Qiagen). RNA gel blots were performed as described previously (Trevaskis et al., 2003). cDNA was prepared for qRT-PCR by using an oligo(T) primer (T18[G/C/A]) to prime first-strand complementary DNA (cDNA) synthesis from 1-5 μg of total RNA with SuperScript III reverse transcriptase enzyme (Invitrogen). qRT-PCR was performed on a Rotor-Gene 3000 real-time cycler (Corbett Research). The primers used for *ACTIN* have been described previously (Trevaskis et al., 2006) and additional primers are detailed in Supplemental Table 4. qRT-PCR was performed using Platinum Taq DNA polymerase (Invitrogen). Cycling conditions were 4 minutes at 94°C, 40 cycles of 10 s at 95°C, 15 s at 60°C, and 20 s at 72°C, followed by a melting-curve program (72°C–95°C with a 5-s hold at each temperature). Fluorescence data were acquired at the 72°C step and during the melting-curve program. Expression levels of genes of interest were calculated relative to *ACTIN* using the comparative quantification analysis method (Rotogene-5; Corbett Research), which takes into account the amplification efficiency of each primer set. Data presented are averages of a minimum of three biological replicates unless stated otherwise and the error bars show SE.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation of leaf tissue was performed as described by Oliver et al. (2009) using the third leaf from non-vernalized or post-vernalized plants. Post-vernalized plants were derived from seeds that had been germinated for seven weeks under vernalizing conditions (4°C), and then transferred to normal glasshouse conditions and grown to the third leaf stage. The results shown are the mean of two biological replicate experiments. Sequences for primers used in ChIP experiments are listed in Supplemental Table 4.

Plant Transformation

Over-expression constructs were made by introducing a full-length *HvOS2* cDNA into a GATEWAY (Invitrogen) adapted cloning vector described previously (Hemming et al. 2008), which uses the maize *UBIQUITIN* promoter (Christensen et al. 1992) to drives transgene expression. RNAi constructs were made using the GATEWAY cloning system; the hairpin cassette from HELLSGATE12 (Wesley et al. 2001) was fused to the maize ubiquitin promoter and placed in the in the pWBVEC8 binary vector backbone (Wang et al. 1998). A map of the resulting vector (pSTARGATE) can be found at http://www.pi.csiro.au/RNAi/vectors.htm. Barley plantswere transformed using *Agrobacterium* transformation of excised embryos of the variety "Golden Promise" (Tingay et al., 1997; Matthews et al., 2001). Golden Promise is as spring barley that flowers without vernalization (genotype *HvVRN1-1, ΔHvVRN2*), and is photoperiod insensitive. T1 and T2 plants were screened for segregation of the transgene using primers that amplify the hygromycin selectable marker gene. Expression analysis was carried out on plants hemizygous or homozygous for the transgene and sibling null control lines which did not inherit the transgene.

Microarray analysis

RNA was extracted using the method of Chang et al. (1993) and then further purified using RNeasy columns (Qiagen). Probe synthesis, labelling, hybridisation to the Barley1 Gene chip (Close et al., 2004) and RNA quality was assessed at the Australian Genome Research Facilities (AGRF; Melbourne, VIC, Australia), following the manufacturer's recommendations (Affymetrix, Santa Clara, CA). Microarray analyses were performed on 3 biological replicates of each sample. The

resulting dataset was analysed in R v2.7.0 and analysed using packages from Bioconductor (Gentleman et al., 2004, http://www.bioconductor.org/), using default settings. Normalisation was carried out by Robust Multichip Analysis (RMA) and differentially expressed genes were identified using the LIMMA package (Linear Models for Microarray Data; (Smyth, 2005)). Genes with p-values higher than 0.01 or with a change in gene expression lower than 1.5-fold were excluded from further analysis. Microarray data has been deposited in the Plant Expression Database (www.plexdb.org), BB XX.

Microscopy and Image analysis

Leaf segments taken from positions at 33% and 66% of the total length of the leaf were fixed at room temperature in 70% ethanol for at least 2h, then dehydrated to 100% ethanol in 10% steps (30 min each step). 100% ethanol was replaced twice and the tissue was critical point dried with CO_2 and mounted on double-sided carbon tabs attached to SEM stubs, adaxial side up. Tissue was then viewed uncoated with a 4 quadrant backscattered electron detector in a Zeiss EVO LS15 SEM. Tissue was viewed using 20 kV accelerating voltage under variable pressure mode, with 10 Pa chamber pressure. Images of the tissue were taken for analysis using the analySIS LS Professional (v2.6). The length of bulliform cells (Wenzel et al., 1997) were measured manually with the line tool.

Sequence Database Searches

All sequence database searches (nucleotide and protein) were performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic Analysis

Alignments of the full nucleotide coding sequences were performed using MUSCLE v.3.6 (Edgar, 2004) and were edited using the BioEdit interface (v. 7.0.9.0) (Hall, 1999), (Supplemental Fig. S1B,D). Phylogenetic analyses were conducted in MEGA4, using the Neighbor-Joining method (default settings) (Saitou and Nei, 1987). Bootstrap values were calculated using 10000 replicates.

Statistical analysis

All statistical analysis was carried out using GenStat 11th Ed. (Payne, 2008) unless specified otherwise.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic relationships between *ODDSOC-like* genes and other plant MADS box genes.

Supplemental Figure S2. Alignments of cDNA and predicted protein sequences.

Supplemental Figure S3. Quantitative RT-PCR analysis of gene expression in *HvOS2* over-expression lines.

Supplemental Figure S4. Phenotypes and expression levels of *HvOS2* in RNAi transgenic plants.

Supplemental Figure S5. Quantitative RT-PCR analysis of *HvOS2* gene expression during development in leaf and crown tissue.

Supplemental Figure S6. Quantitative RT-PCR analysis of *TmOS2* gene expression in the Δ*VRN1* mutant grown in long days.

Supplemental Figure S7. Selected data from low temperature stress microarray experiment (cv. Dicktoo).

Supplemental Figure S8. Leaf appearance rate and final leaf number in *HvOS2* over-expression lines versus null sibling control lines.

Supplemental Figure S9. Images of transgenic plants over-expressing *HvOS2* and wild-type siblings with or without GA treatment.

Supplemental Figure S10. Selected data from microarray analysis of gene expression during barley development (cv. Morex).

Supplemental Table S1. Nearest Arabidopsis homologues of *HvOS1* and *HvOS2*, BlastP.

Supplemental Table S2. Nearest Arabidopsis homologues of *HvOS1* and *HvOS2*, BlastN.

Supplemental Table S3. Microarray results for comparison of *HvOS2* over-expression line versus a null sibling control.

Supplemental Table S4. Primers used in this study.

Acknowledgments

We thank our colleagues Steven Swain and Peter Chandler, for helpful discussions and suggestions for improvement of the manuscript. We thank Sarah Fieg and Sandra Stops for technical support and K. Murai for the kind donation of the *mvp* seeds. We also thank Million Tadege for providing cDNA clones used in this study. This work was supported by the CSIRO Division of Plant Industry, the CSIRO Office of the Chief Executive and by a PhD scholarship to Aaron Greenup from the College of Sciences at the Australian National University.

Footnotes

The author(s) responsible for distribution of materials integral to the findings presented in this article in accordance with the Journal policy described in the Instructions for Authors (http://www.plantphysiol.org) is: Ben Trevaskis (Ben.Trevaskis@csiro.au).

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Figure legends

Figure 1. *HvOS1* and *HvOS2* are members of a grass specific class of MADS box genes. A, Diagrammatic representation of the syntentic region in rice and *Brachypodium distachyon* that contains the *ODDSOC-like* genes (*OsMADS51*, *BdOS1* and *BdOS2*), and the corresponding barley Unigene numbers and map locations. Arrows indicate direction of transcription. B, Phylogenetic relationships between the *ODDSOC-like* genes of rice (*OsMADS51*), maize (*ZmOS-like*), sorghum (*SbOS-like*), barley (*HvOS1* and *HvOS2*), wheat (*TaAGL33*, *TaAGL41* and *TaAGL42*) and *Brachypodium distachyon* (*BdOS1-like* and *BdOS2-like*) based on a sequence alignment of the coding sequence for each gene.

Figure 2. Vernalization-induced changes in *HvOS1* and *HvOS2* transcript levels.
A, Expression of *HvOS1* and *HvOS2* in barley (cv. Sonja) seedlings germinated in darkness at 20° (non-vernalized = NV, white bar, n = 4) versus seedlings germinated in darkness at 4 degrees for 49 days (vernalized = V, black bar, n = 3), harvested at an equivalent stage of development. B, Expression levels of *HvOS2* in fully-expanded 1st leaves of non-vernalized plants (NV, white bar, n = 3) versus vernalized plants (post-vernalization = PV, black bar, n = 3), harvested in long-days at the 2 leaf stage, 10 days after the end of vernalization. C, Expression levels of *HvOS2* in shoot apices (30-50 individual apices) from non-vernalized plants (0) or after 2, 5 or 9 weeks vernalization (n = 2). Expression levels (A-C) were assayed by qRT-PCR and are shown relative to *ACTIN*. Error bars show SE (A and B) or range. Asterisks indicate P values of ANOVA: * $P < 0.05$, *** $P < 0.001$.

Figure 3. Analysis of histone modifications at *HvOS2* during vernalization
A, Relative abundance of H3K27me3 at the start of transcription for *HvOS2* in non-vernalized plants (NV, black bars) and vernalized plants (V, white bars) (cv. Sonja). B, Relative abundance of H3K4me3 at the start of transcription for *HvOS2* in non-vernalized plants (NV, black bars) and vernalized plants (V, white bars) (cv. Sonja) Error bars show SD.

Figure 4. Expression of *ODDSOC2* in different genotypes of wheat and barley

A, *HvOS2* expression levels in non-vernalized (-) versus vernalized plants (+) (2 weeks old, grown in long days) from different barley cultivars, including 3 spring barleys that flower without vernalization: Morgenrot (lanes 1, 2), Randolph (3,4), Malta (5,6) and three vernalization-responsive winter barleys Sonja (7,8), Hudson (9,10), Mirra (11,12). Expression was assayed by high-stringency hybridization of RNA gel blots with a *HvOS2*-specific riboprobe. Ethidium bromide staining of ribosomal RNA is shown as a loading comparison. B, *HvOS2* expression levels assayed by qRT-PCR in RNA from barley seedlings of a doubled haploid barley population (Sloop × Halcyon). Expression of *HvOS2* was assayed in individual lines, relative to *ACTIN*, the average expression levels of the different *HvVRN1* genotypic classes were compared (*VRN1* $n = 22$, *VRN1-1* $n = 19$). C, Relative expression levels of *TmOS2-like* (*TmAGL33*) in the *TmVRN1* deletion mutant (Δ*VRN1*) (white bars) versus the wild type parent strain (black bars). Expression was assayed in vernalized (V) ($n = 4$), non-vernalized (NV) ($n = 4$) seedlings and in the leaves of plants grown for 1 week or 3 weeks (WT $n = 3$, Δ*VRN1* $n = 2$) in short days (SD) post-vernalization (PV). Expression is shown relative to *ACTIN*. D, Relative expression levels of *TmVRN1* as in the conditions described in C. Error bars show SE. Asterisks indicate P values of ANOVA: ***, P , <0.001, ND, not detected.

Figure 5. Phenotypes of transgenic plants that over-express *HvOS2*.

A, Average days to head emergence (heading date) of transgenic barley lines over-expressing *HvOS2* (black), versus sibling null segregant controls (plants from same transgenic line that did not inherit the transgene) (WT, white). B, Transgenic barley plants over-expressing *HvOS2* versus null segregants at the 4th leaf stage (left) and apex images from the same stage (right). DR indicates 'double ridges' the first sign of floral development. L indicates leaf primordia. C, Average length of the 1st and 3rd leaves of plants over-expressing *HvOxO2* (*OxHvOS2-20*) (black) versus the null controls (white) at the 6th leaf stage, (WT, $n = 5$), 5 (*OxHvOS2-20*, $n = 5$). D, Scanning Electron Microscopy (SEM) images of epidermal cells from the adaxial surface of mature leaves (1st and 2nd leaves). E, Average length of bulliform cells on the adaxial surface of mature leaves (1st and 2nd leaves) from plants over-expressing *HvOxO2* (*OxHvOS2-20*) (black) and wild-type siblings (white). Cell lengths were measured from SEM images taken at position 33% and 66% blade lengths. L1: WT $n = 367$ (cells) and *OxHvOS2-20,* $n = 467$, L2: WT $n = 344$ and *OxHvOS2-20* $n = 478$.

F, Average head and internode lengths of the primary tiller of plants over-expressing *HvOxO2* (black) versus null segregants (white) (P1-peduncular internode, P-1 internode: below peduncle internode, P-2 below P-1, and P-3 below P-2) $n = 15$. Error bars show SE. Asterisks indicate P values of ANOVA: ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Influence of vernalization on the expression of *FPF1-like* genes in short or long days.

Expression of *HvFPF1-like1* (HU14G14r) (A) and *HvFPF1-like2* (Contig18182) (B) in the fully expanded 2nd leaf (harvested at the 3rd leaf stage), non-vernalized (white) versus vernalized plants (black), grown in long days (LD) or short days (SD). Expression was assayed by qRT-PCR and is shown relative to *ACTIN*. Error bars show SE. Asterisks indicate P values of ANOVA test: * $P < 0.05$ (min. of 3 biological repeats).

Figure 7. An extended model of the molecular genetic network that controls vernalization-induced flowering in temperate cereals.

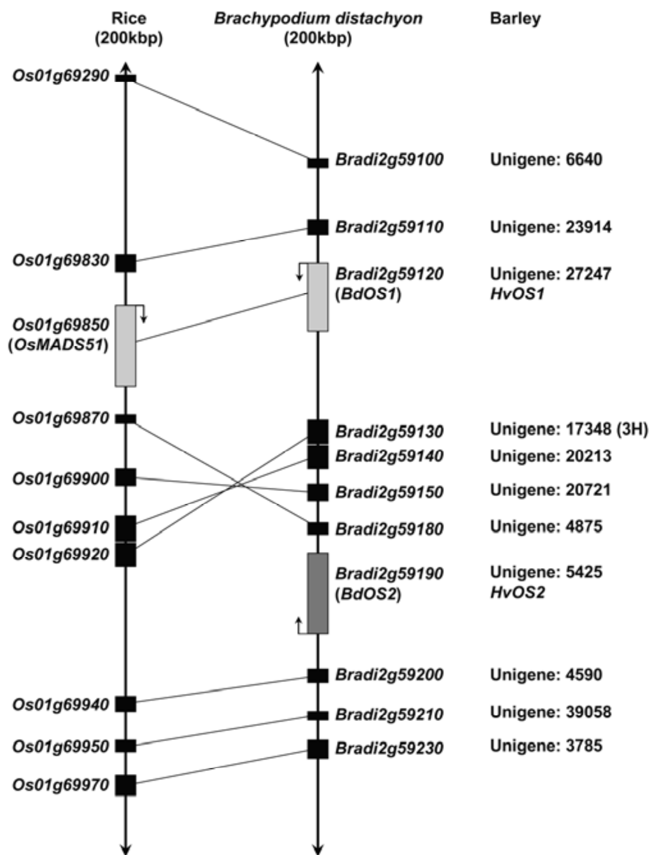
Low-temperatures (cold) can transiently down-regulate *ODDSOC2*. Prolonged cold (vernalization) causes stable activation of *VRN1*. After vernalization, *VRN1* down-regulates *ODDSOC2* (*OS2*), directly or indirectly. Consequently, *FLOWERING PROMOTING FACTOR1-like* genes (*FPF1s*) are de-repressed. *VRN1* also down-regulates *VRN2* and allows activation of *FT1* by long days (see Trevaskis et al 2007, Distelfeld et al. 2009). Expression of *FPF1s* is induced as *FT1* activity increases, and this promotes the transition to reproductive development at the shoot apex and cell elongation in the stem.

Table I. Non-redundant blastp results for HvOS2

Protein Description	Accession	Organism	Identity	BLAST Score	E-value
TaAGL33	ABF57950	*T. aestivum*	93% (147/158)	297	2e-79
TaAGL41	ABF57941	*T. aestivum*	84% (126/149)	255	1e-66
TaAGL42	ABF57942	*T. aestivum*	73% (114/155)	225	1e-57
OsMADS51 (*Os01g69850*)	NP_001045235	*O. sativa*	74% (111/149)	224	2e-57
Hypothetical protein (*Sb03g044170*)	XP_002456860	*S. bicolor*	70% (110/157)	219	6e-56
Hypothetical protein (LOC100272251)	NP_001140218	*Z.mays*	69% (108/155)	197	2e-49

Table II. Top 5 up-regulated and down-regulated genes in *HvOS2* over-expression line

Probe Set	Best match	FC	P-Value
Up-regulated			
Contig5058_x_at	*RNase S-like* (*T. aestivum*)	37.6	5.3E-09
Contig5059_s_at	*RNase S-like* (*T. aestivum*)	36.8	3.9E-09
Contig5185_at	*rsh1*, *RNase S-like* (*H. vulgare*)	16.2	1.1E-07
Contig12031_at	*HvODDSOC2 (H. vulgare)*	15.4	7.8E-10
Contig1568_x_at	THION9 - Plant thionin family protein (*O. sativa*)	8.2	5.5E-04
Down-regulated			
Contig3810_at	*Galactinol synthase (T. aestivum)*	-2.9	1.6E-05
HVSMEm0003G16r2_at	*cytochrome P450* (*O. sativa*)	-3.4	5.7E-03
HVSMEb0010F06r2_at	No description	-3.6	3.1E-06
Contig18182_at	*FLOWERING PROMOTING FACTOR1 -like* (*A. thaliana*)	-3.7	3.8E-06
HU14G14r_s_at	*FLOWERING PROMOTING FACTOR1 -like* (*A. thaliana*)	-6.0	5.2E-05



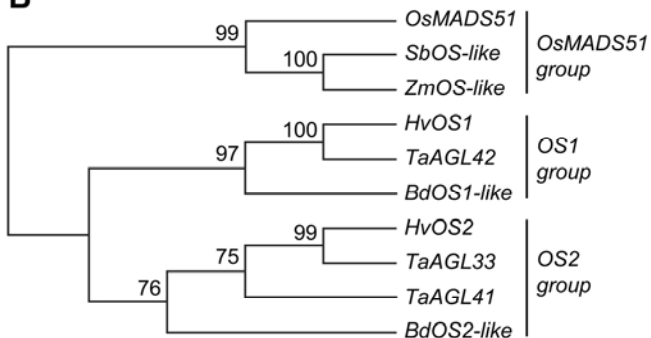


Figure 1. *HvOS1* and *HvOS2* are members of a grass specific class of MADS box genes. A, Diagrammatic representation of the syntentic region in rice and *Brachypodium distachyon* that contains the *ODDSOC-like* genes (*OsMADS51*, *BdOS1* and *BdOS2*), and the corresponding barley Unigene numbers and map locations. Arrows indicate direction of transcription. B, Phylogenetic relationships between the *ODDSOC-like* genes of rice (*OsMADS51*), maize (*ZmOS-like*), sorghum (*SbOS-like*), barley (*HvOS1* and *HvOS2*), wheat (*TaAGL33*, *TaAGL41* and *TaAGL42*) and *Brachypodium distachyon* (*BdOS1-like* and *BdOS2-like*) based on a sequence alignment of the coding sequence for each gene.

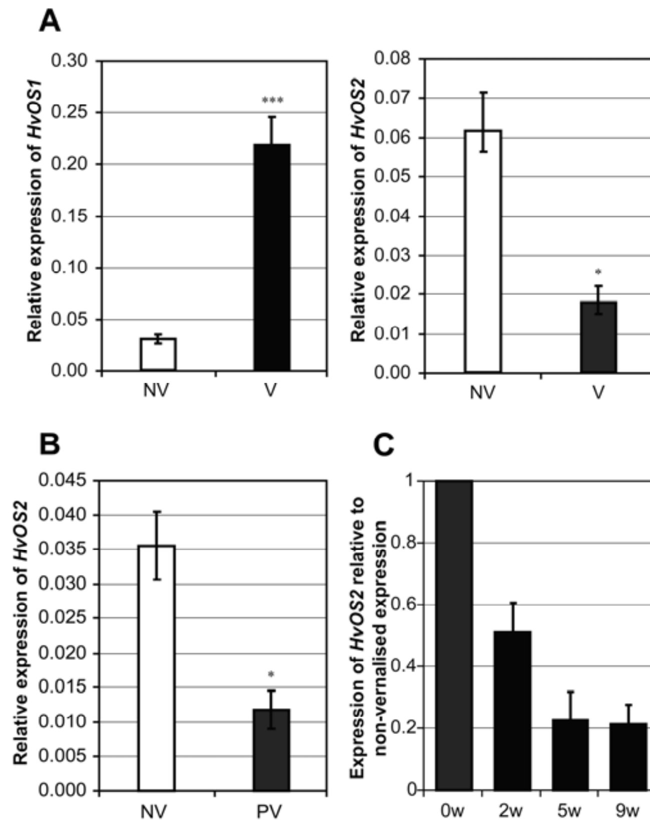


Figure 2. Vernalization-induced changes in *HvOS1* and *HvOS2* transcript levels.

A, Expression of *HvOS1* and *HvOS2* in barley (cv. Sonja) seedlings germinated in darkness at 20° (non-vernalized = NV, white bar, n = 4) versus seedlings germinated in darkness at 4 degrees for 49 days (vernalized = V, black bar, n = 3), harvested at an equivalent stage of development. B, Expression levels of *HvOS2* in fully-expanded 1st leaves of non-vernalized plants (NV, white bar, n = 3) versus vernalized plants (post-vernalization = PV, black bar, n = 3), harvested in long-days at the 2 leaf stage, 10 days after the end of vernalization. C, Expression levels of *HvOS2* in shoot apices (30-50 individual apices) from non-vernalized plants (0) or after 2, 5 or 9 weeks vernalization (n = 2). Expression levels (A-C) were assayed by qRT-PCR and are shown relative to *ACTIN*. Error bars show SE (A and B) or range. Asterisks indicate P values of ANOVA: * $P < 0.05$, *** $P < 0.001$.

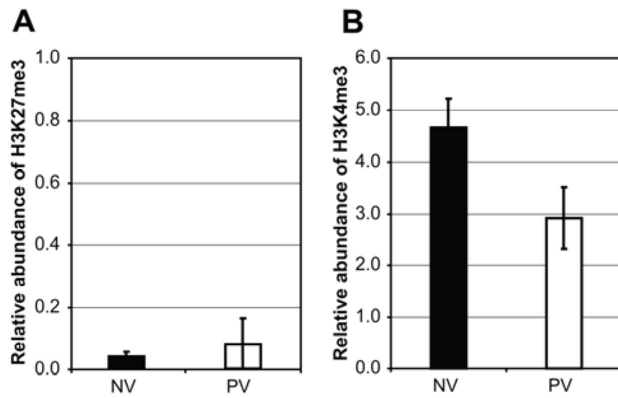


Figure 3. Analysis of histone modifications at *HvOS2* during vernalization

A, Relative abundance of H3K27me3 at the start of transcription for *HvOS2* in non-vernalized plants (NV, black bars) and vernalized plants (V, white bars) (cv. Sonja). B, Relative abundance of H3K4me3 at the start of transcription for *HvOS2* in non-vernalized plants (NV, black bars) and vernalized plants (V, white bars) (cv. Sonja).

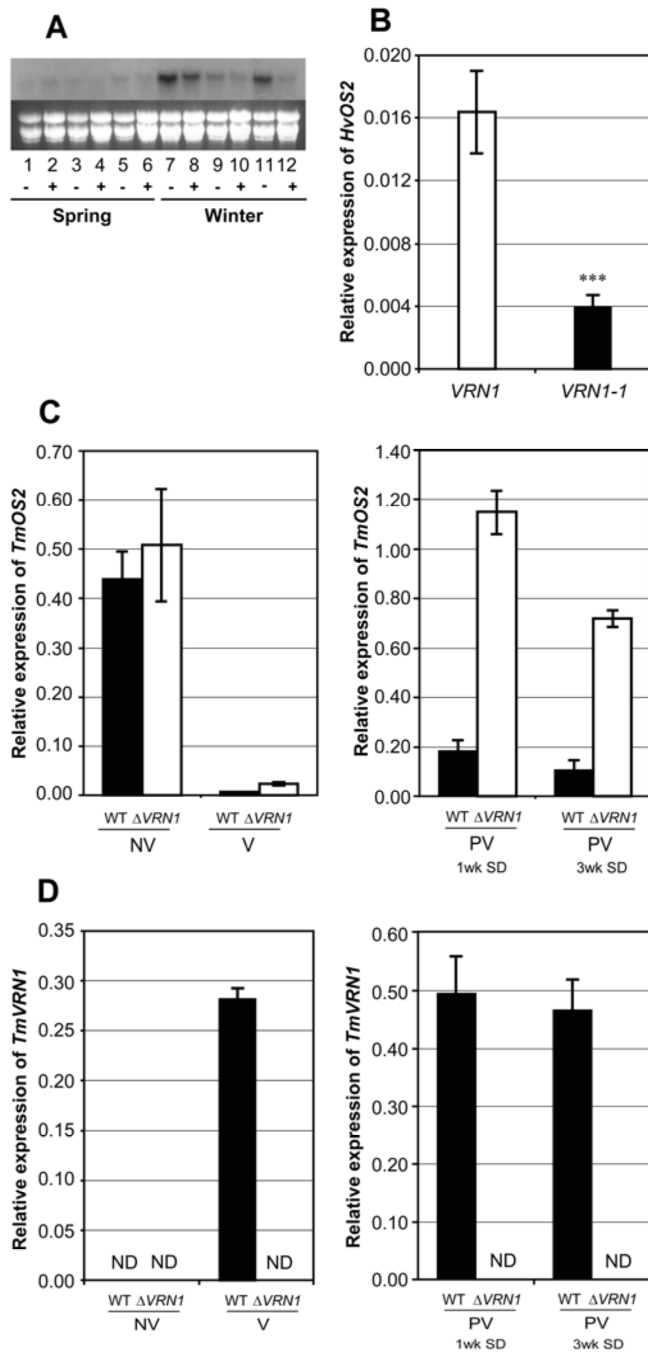


Figure 4. Expression of *ODDSOC2* in different genotypes of wheat and barley
A, *HvOS2* expression levels in non-vernalized (-) versus vernalized plants (+) (2 weeks old, grown in long days) from different barley cultivars, including 3 spring barleys that flower without vernalization: Morgenrot (lanes 1, 2), Randolph (3,4), Malta (5,6) and three vernalization-responsive winter barleys Sonja (7,8), Hudson (9,10), Mirra (11,12). Expression was assayed by high-stringency hybridization of RNA gel blots with a *HvOS2*-specific riboprobe. Ethidium bromide staining of ribosomal RNA is shown as a loading comparison. B, *HvOS2* expression levels assayed by qRT-PCR in RNA from barley seedlings of a doubled haploid barley population (Sloop × Halcyon). Expression of *HvOS2* was assayed in individual lines, relative to *ACTIN*, the average expression levels of the different *HvVRN1* genotypic classes were compared (*VRN1* n = 22, *VRN1-1* n = 19). C, Relative expression levels of *TmOS2-like* (*TmAGL33*) in the *TmVRN1* deletion mutant (Δ*VRN1*) (white bars) versus the wild type parent strain (black bars). Expression was assayed in vernalized (V) (n = 4), non-vernalized (NV) (n = 4) seedlings and in the leaves of plants grown for 1 week or 3 weeks (WT n = 3, Δ*VRN1* n = 2) in short days (SD) post-vernalization (PV). Expression is shown relative to *ACTIN*. D, Relative expression levels of *TmVRN1* as in the conditions described in C. Error bars show SE. Asterisks indicate P values of ANOVA: ***, P , <0.001, ND, not detected.

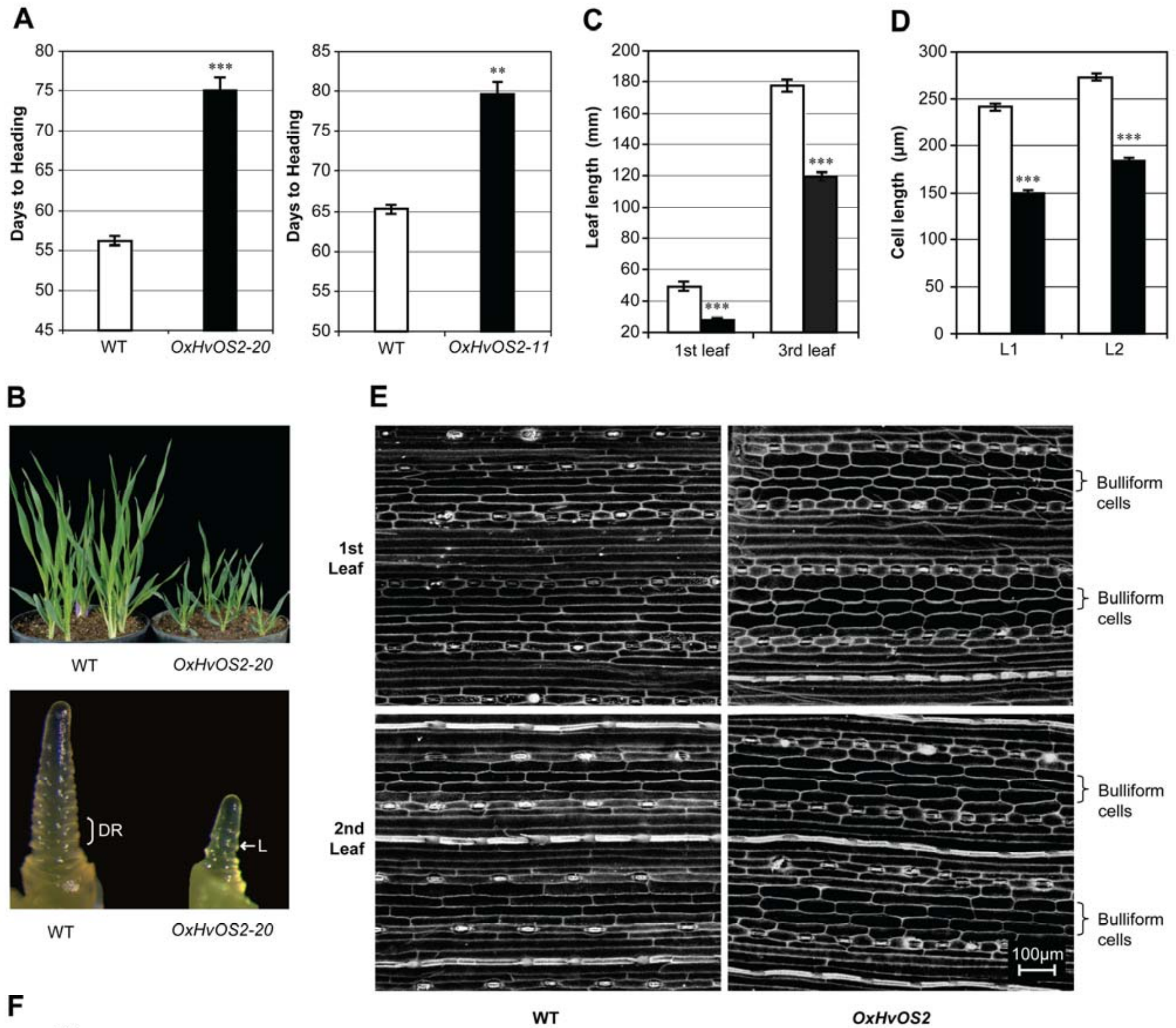


Figure 5. Phenotypes of transgenic plants that over-express *HvOS2*. A, Average days to head emergence (heading date) of transgenic barley lines over-expressing *HvOS2* (black), versus sibling null segregant controls (plants from same transgenic line that did not inherit the transgene) (WT, white). B, Transgenic barley plants over-expressing *HvOS2* versus null segregants at the 4th leaf stage (left) and apex images from the same stage (right). DR indicates 'double ridges' the first sign of floral development. L indicates leaf primordia. C, Average length of the 1st and 3rd leaves of plants over-expressing *HvOxO2* (*OxHvOS2-20*) (black) versus the null controls (white) at the 6th leaf stage, (WT, n = 5), 5 (*OxHvOS2-20*, n = 5). D, Scanning Electron Microscopy (SEM) images of epidermal cells from the adaxial surface of mature leaves (1st and 2nd leaves). E, Average length of bulliform cells on the adaxial surface of mature leaves (1st and 2nd leaves) from plants over-expressing *HvOxO2* (*OxHvOS2-20*) (black) and wild-type siblings (white). Cell lengths were measured from SEM images taken at position 33% and 66% blade lengths. L1: WT n = 367 (cells) and *OxHvOS2-20,* n = 467, L2: WT n = 344 and *OxHvOS2-20* n = 478. F, Average head and internode lengths of the primary tiller of plants over-expressing *HvOxO2* (black) versus null segregants (white) (P1-peduncular internode, P-1 internode: below peduncle internode, P-2 below P-1, and P-3 below P-2) n = 15. Error bars show SE. Asterisks indicate P values of ANOVA: ** $P < 0.01$, *** $P < 0.001$.

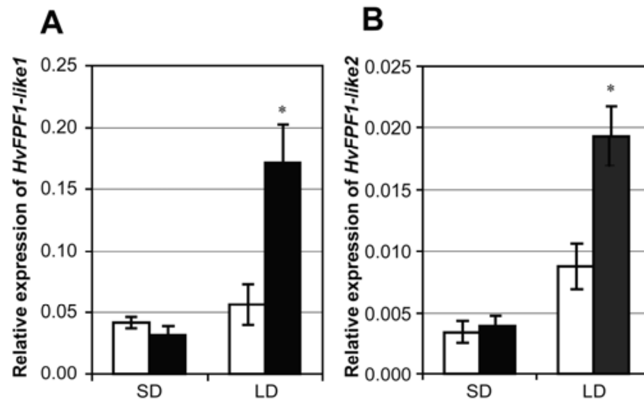


Figure 6. Influence of vernalization on the expression of *FPF1-like* genes in short or long days.

Expression of *HvFPF1-like1* (HU14G14r) (A) and *HvFPF1-like2* (Contig18182) (B) in the fully expanded 2nd leaf (harvested at the 3rd leaf stage), non-vernalized (white) versus vernalized plants (black), grown in long days (LD) or short days (SD). Expression was assayed by qRT-PCR and is shown relative to *ACTIN*. Error bars show SE. Asterisks indicate P values of ANOVA test: * $P < 0.05$ (min. of 3 biological repeats).

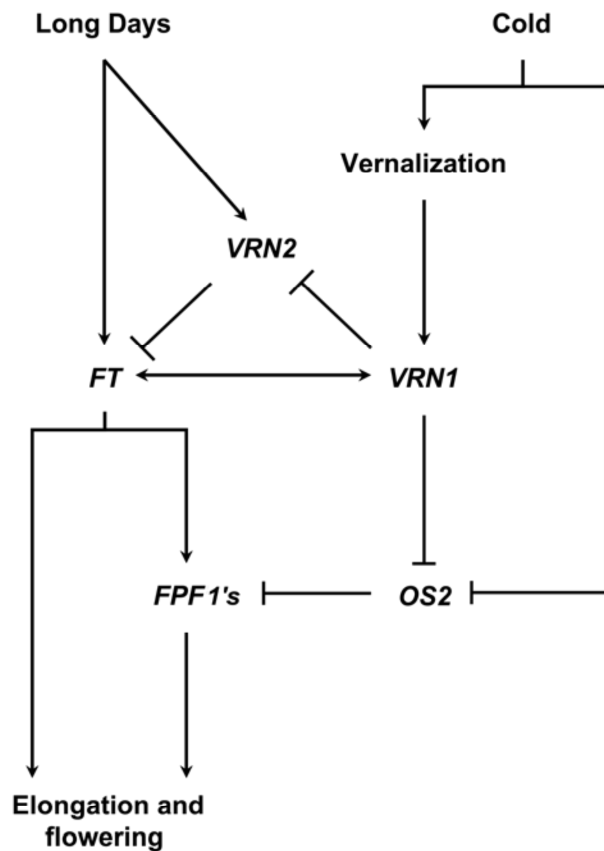


Figure 7. An extended model of the molecular genetic network that controls vernalization-induced flowering in temperate cereals.

Low-temperatures (cold) can transiently down-regulate *ODDSOC2*. Prolonged cold (vernalization) causes stable activation of *VRN1*. After vernalization, *VRN1* down-regulates *ODDSOC2* (*OS2*), directly or indirectly. Consequently, *FLOWERING PROMOTING FACTOR1-like* genes (*FPF1s*) are de-repressed. *VRN1* also down-regulates *VRN2* and allows activation of *FT1* by long days (see Trevaskis et al 2007, Distelfeld et al. 2009). Expression of *FPF1s* is induced as *FT1* activity increases, and this promotes the transition to reproductive development at the shoot apex and cell elongation in the stem.