Supplemental data

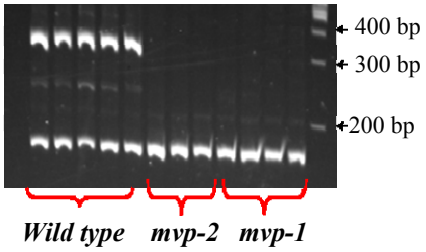
"Freezing tolerance and flowering regulation in cereals: the *VRN-1* connection"

Table S1. Primers used for quantitative RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Prod. bp[1]	Eff. (%)[2]
CBF12	GTCCCACTCCCACTCACAG	ACATGTCGTGGCACAATGC	74	95
CBF14	CCACCAAATATGGGAGGAAA	GCTTTCACAATGAACGAGCA	73	92
CBF15	CATGTTCGAGCTGGATATGTCCGGGG	GGGAACAGCTTCGGTTTGTTCATGC	213	100
CBF16	GCGGCATGCCTCCAACAGCGCAG	ACGTGCCCAGGTCCATCTCCCCG	200	93
CBF13	TGGACATCGACATGTTCAGGCTTG	CAGAGCAGAATCAGATGGGGAATC	214	91
CBF10	TGTTCAGTAGGCTTGACTTGTTCCCG	GCAGAATCGGCTACAAGCTCCAG	180	86
CBF3	GCGGTGGACACCGATATGTTCAG	GCGAGGGGAATTATCGACTGTAC	204	87
CBF17	GAGTTCGACTTGGAGCTGGA	CGAAATCCGCGTAGTAGGAG	67	96
CBF9	CCAGCAGCAGCAGATCATT	CGCCGGAAGACATGTAAAAC	65	92
CBF4	GCTGTTCTCCATGTCGTCAG	GTAGTACGACCCGGCAACC	82	94
CBF2	GTCCATCACCTCCAACGACT	GCGCCAAGTTTGCGTAGTA	89	86
COR14b	GAGCGACTCCTGCTAACGAC	CTACCGCCTCCTGTACCTTG	135	96
ACTIN	ACCTTCAGTTGCCCAGCAAT	CAGAGTCGAGCACAATACCAGTTG	91	98
TEF1	GCCCTCCTTGCTTTCACTCT	AACGCGCCTTTGAGTACTTG	91	99

[1] PCR product size in base pairs. [2] Primers efficiency.

Figure S1. Dominant *VRN-1* molecular marker used to classify segregating plants into homozygous mutants (*mvp*/*mvp*) and heterozygous plus homozygous wild type (*Mvp*/-) classes. The borders of the deletion are currently unknown.



Methods for Fig. S1: This dominant marker uses three primers in the PCR reaction. Primers MVP_F-18 and MVP_R-22 amplify a 172-bp band in both the mutant and non-mutant lines that is used as an amplification control, whereas primers MVP_F-18 and MVP_R-23 amplify a 340-bp band that is only present in the non-mutant lines. PCRs were carried out in a 20 µl volume using 500 p moles each of MVP_R22 and MVP_R23, and 750 p moles of MVP_F18.

Primers:

MVP_F-18: 5′-AGCCACAAGAACCGGGACTA-3′
MVP_R-22: 5′-ATTCAAGCCCCAATGTTCTC-3′
MVP_R-23: 5′-CCCAAACTTTGCGGTGTATC-3′

PCR conditions:

40 cycles of: 94^{o}C-20 s, 60^{o}C-20 s, 72^{o}C-15 s.

Figure S2: Apices of *Mvp-2/-* and *mvp-2/mvp-2* plants before and after cold acclimation in the freezing tolerance test described in Figure S3 (-12 °C). Apices were photographed after 35 days at 20 °C and again after additional 18 days of cold hardening at 4 °C before the freezing experiment. The apices from the *mvp-2/mvp-2* mutant plants were at the vegetative stage whereas those from *Mvp-2/-* plants were at the double ridge stage (dr).

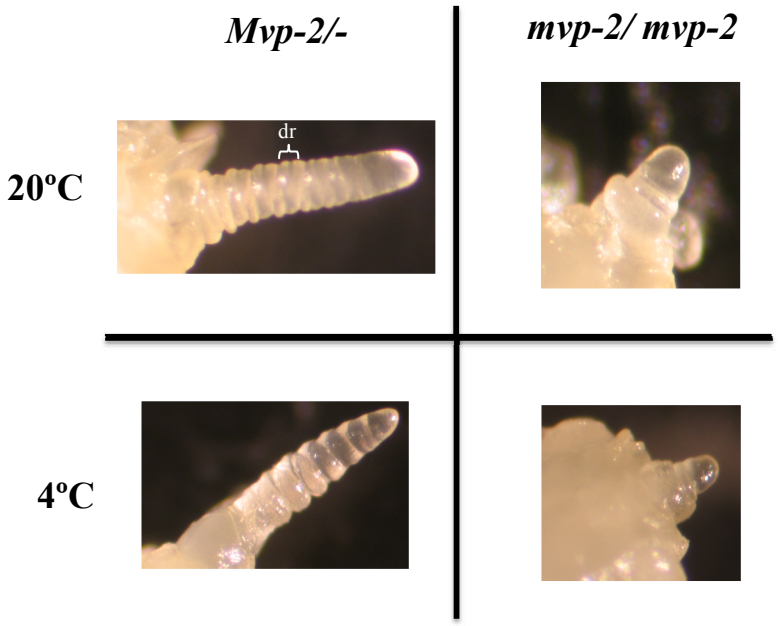
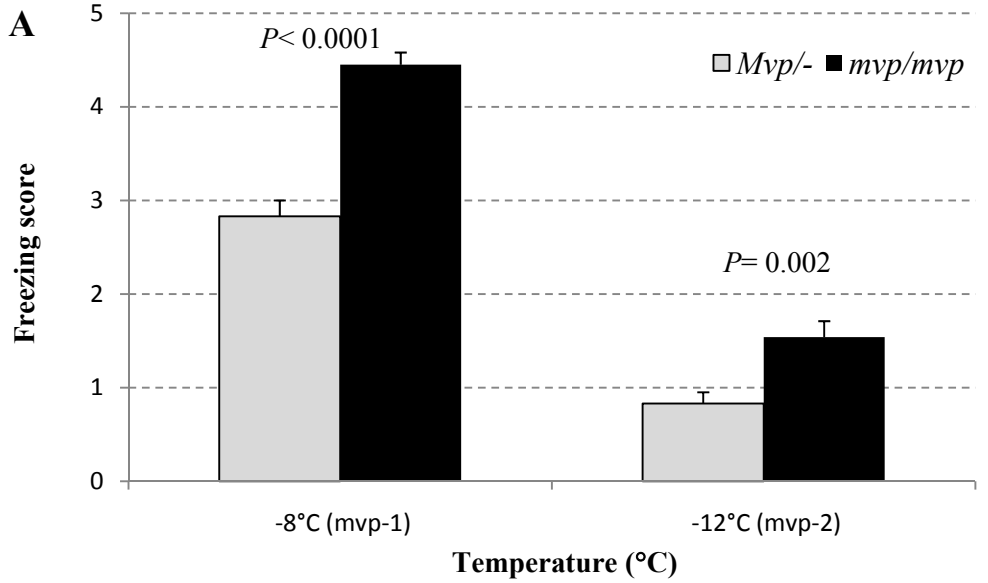
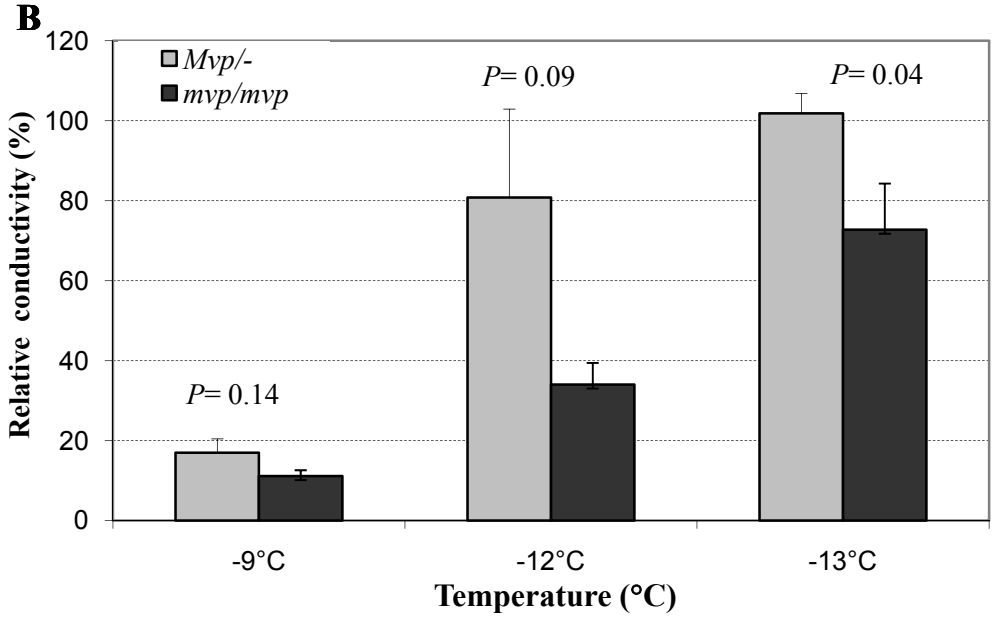


Figure S3. **A)** Average freezing scores using a scale from 0= dead plants to 5= undamaged plants. The -8 ^{o}C experiment included 22 *mvp-1/mvp-1* homozygous mutants (black bars) and 35 *Mvp-1/-* plants (gray bars) carrying at least one functional *VRN-1* copy. The -12 ^{o}C experiment used 24 *mvp-2/mvp-2* homozygous mutants and 65 *Mvp-2/-* plants. Plants were 35 days old before the cold acclimation treatment. *P* values correspond to ANOVAS comparing *mvp/mvp* (black bars) and *Mvp/-* (gray bars) plants. **B)** Average relative conductivity (ion leakage) of leaf segments from *mvp-2/mvp-2* and *Mvp-2/-* plants. Error bars represent standard errors of the means (SE) based on 9 replications per genotype / temperature combination.





Material and Methods used for Figure S3:

A) Regrowth. Seeds were germinated in Petri dishes and seedlings were transplanted to wooden boxes (42 cm long x 30 cm wide x 14 cm high) with 9 cm of soil depth. Soil consisted of 4:1 soil to sand. Plants were grown for 35 days in a Conviron growth chamber using a light intensity of 260 μmol m^{-2} s^{-1}, constant temperature of 20°C, and 75 % relative humidity. Plants were genotyped with the marker described above in Fig. S1.

After 35 days temperature was decreased 4°C per day until the temperature reached 4°C, which was then held constant for 18 d. Both growth and cold hardening were carried out under long day conditions 16/8 hours (day/night). This was followed by acclimation at -2°C of 6h and 17h (separated by +2°C for 7 hours), and at -4°C for 22 h. Ice nucleation was induced by spraying the leaves with water. Temperature was then lowered 1°C/h to the target temperatures of -9°C, -12°C, and -13°C, which were held for 24 h.

After the freezing treatment temperature was increased 2°C per h to 17°C under a light intensity of 260 μmol m^{-2} s^{-1}, a 16 h/8 h light/dark photoperiod cycle and 75% RH. After two days the leaves were cut several cm above the soil. Regrowth was scored after 14 days of recovery.

B) Electrolyte leakage assay: Electrolyte leakage assays from plants treated at -9, -12 and -13 ºC (Fig. S3B) utilized the middle one-third section of the oldest leaf. After removal from the plant, the weight and length of this leaf section was determined. Leaf sections were rinsed with DI-H_2O, and then incubated overnight in 10 ml of DI-H_2O in tubes, during which time they were gently shaken.

Maximum ion leakage was determined by boiling three independent *mvp*/*mvp* homozygous mutant class leaf samples and three independent *Mvp*/- leaf samples for 20 minutes. Conductance was measured using a MultiSample Conductometer (Mikro KKt., Hungary) in an average of 9 plants per genotype temperature combination. Relative conductance was calculated using the equation, Relative conductance = (Measured conductance – Average conductance of di-H_2O)/ (Average maximum conductance - Average conductance of di-H_2O)* 100.

Fig. S4. Quantitative RT-PCR analysis of transcript levels of the *CBF* genes present at the *FR-2* locus. Values are expressed relative to the *TEF1* endogenous control (*COR14b* transcript levels are included as reference). Leaf samples were collected from 8-weeks old plants 8 h after transferring plants from 20 °C to 4 ºC. Values in the Y axes were normalized and calibrated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The same calibrator was used for all genes to ensure scales are comparable across genes. Homozygous *mvp-1/ mvp-1* plants are indicated in black and those of *Mvp-1*/- plants carrying 1 or 2 functional *VRN-1* copies in gray. Values are averages of 10 biological replications ± SE of the means. The inset shows the *CBF* genes with relatively lower transcript levels using a different scale. *P* values were calculated using ANOVA of log(n+1)transformed values *: $P<0.05$ and **: $P<0.01$.