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## Identification of Quantitative Trait Loci and a candidate locus for freezing tolerance in controlled and outdoor environments in the overwintering crucifer *Boechnera stricta*

Jae-Yun Heo<sup>1</sup>, Dongsheng Feng<sup>2</sup>, Xiaomu Niu<sup>2</sup>, Thomas Mitchell-Olds<sup>3</sup>, Peter H. van Tienderen<sup>4</sup>, Dwight Tomes<sup>2</sup>, and M. Eric Schranz<sup>1,\*</sup>

<sup>1</sup>Biosystematics Group, Wageningen University & Research Center, Wageningen, the Netherlands <sup>2</sup>Pioneer Hi-Bred International, Inc., a DuPont Business, Johnston, USA <sup>3</sup>Duke University, Durham, North Carolina, USA <sup>4</sup>Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, the Netherlands

### Abstract

Development of chilling and freezing tolerance is complex and can be affected by photoperiod, temperature and photosynthetic performance; however, there has been limited research on the interaction of these three factors. We evaluated 108 recombinant inbred lines of *Boechnera stricta*, derived from a cross between lines originating from Idaho and Colorado, under controlled Long-Day (LD), Short-Day (SD) and in an Outdoor Environment (OE). We measured maximum quantum yield of photosystem II, lethal temperature for 50% survival and electrolyte leakage of leaves. Our results revealed significant variation for chilling and freezing tolerance and photosynthetic performance in different environments. Using both single and multi-trait analyses, three main-effect Quantitative Trait Loci (QTL) were identified. QTL on LG3 were SD-specific, whereas QTL on LG4 were found under both LD and SD. Under all conditions, QTL on LG7 were identified, but were particularly predictive for the Outdoor Experiment. The co-localization of photosynthetic performance and freezing tolerance effects supports these traits being co-regulated. Finally, the major QTL on LG7 is syntenic to the Arabidopsis *CBF* locus, known regulators of chilling and freezing responses in *A. thaliana* and other species.

### Keywords

cold-tolerance; photoperiod; natural variation; *Boechnera*; Brassicaceae

## INTRODUCTION

Genetic variation for freezing tolerance is important for understanding both adaptation of species to natural environments and for improving crop performance in stressful conditions. Research over the last decades has demonstrated that complex physiological and biochemical changes occur in a wide range of plant species during cold acclimation,

\*Corresponding author: Dr. M. Eric Schranz. eric.schranz@wur.nl.

including both freezing-tolerant and freezing-sensitive plants (Leinonen, 1996; Hannah et al. 2006; Carvallo et al. 2011). The level of tolerance to freezing in plants is influenced by membrane and cell composition, the accumulation of carbohydrates and adjustments to the photosynthetic apparatus (Sandve et al. 2011). Some low-temperature differentially-regulated genes can be associated with these important changes and can be used for understanding the mechanisms of chilling and freezing tolerance (Thomashow, 2010).

QTL (Quantitative Trait Locus) studied in *Arabidopsis thaliana* and temperate Triticeae species of the grass family have shown QTL regions containing CBF (C-repeat binding factor) genes are associated with their freezing tolerance. Previously, it has been established that three members of the CBF family, CBF1, CBF2 and CBF3, play a key role in the regulation of the transcriptome during cold acclimation (Maruyama et al. 2004; Xu et al. 2011). These CBF genes have been isolated from several herbaceous and woody plant species and different studies have demonstrated their significant role for development of freezing tolerance (Zhang et al. 2004; Skinner et al. 2005; Gamboa et al. 2007; Welling and Palva, 2008; He et al. 2012). CBFs have therefore been used to develop strategies to enhance freezing tolerance in cultivated crops and to understand adaptation to cold environments in native species. However, variation in *CBF* loci does not explain all the quantitative natural variation for freezing tolerance (Gery et al. 2011; Meissner et al. 2013). In addition, the ecological context of *Arabidopsis*, a model species, is often unclear due to their growth in highly disturbed environments.

The genus *Boechera* is closely related to *Arabidopsis* and is being developed as an additional model system to understand plant adaptation. *Boechera stricta* is a genetically tractable, short-lived, overwintering, perennial species. It grows mostly in undisturbed habitats of Western North America, with habitats widely varying in abiotic and biotic conditions. For example, populations can be found across a 2000 m elevation gradient, and this is expected to have an effect on the genetic variation of genes controlling ecologically important traits (Schranz et al. 2009). Previously, a number of genomic resources and a genetic map have been developed for identifying ecologically relevant QTL in *B. stricta*, allowing extensive comparative analyses with *Arabidopsis* (Schranz et al. 2007a; Anderson et al. 2011; Anderson et al. 2014). Here, we utilized these resources to study freezing tolerance in *B. stricta* grown under both controlled and outdoor conditions. In addition, photosynthetic performance can be also associated with freezing tolerance because photosynthesis may be a critical factor for freezing or frost tolerance. However, there has been little research conducted on the genetic regulation of freezing tolerance and photosynthetic performance. Hence, we also analyzed photosynthetic performance by measuring the maximum quantum yield of photosystem II.

In this study, we found significant variation on freezing tolerance and photosynthetic performance to freezing stress conditions in *B. stricta* and also observe that photosynthetic performance may be genetically associated with freezing tolerance. Within the QTL analysis, we have identified three major QTLs, which will be useful for clarifying underlying ecologically important questions on freezing tolerance.

## MATERIALS AND METHODS

### Plant materials

For all experiments we used 108 selected RIL lines and parental genotypes from a population derived from a cross between two highly inbred lines of *B. stricta* (Graham) Al-Shehbaz. The maternal line ‘SAD12’ was collected from Colorado (elevation: 2530m) and the paternal line ‘LTM’ was collected in Montana (elevation: 2390m). The parental sites in Montana and Colorado differ in rainfall, temperature, day length and ecological community (Schrantz et al. 2007b). The F<sub>7</sub> RILs have previously been genotyped and used in mapping and QTL experiments (Anderson et al. 2011).

### Plant growth

Seeds were germinated in petri dishes, and then transferred onto pots with the soil mixture of No.1 and No.3 soil (Jongkind Ground BV) in a 1:2 proportion. Plants were established for three to four weeks, and then used to perform three different freezing stress experiments: two controlled climate chamber experiments differing in photoperiod, Long Day (LD) and Short Day (SD), and an Outdoor Environment (OE). Day-lengths in outdoor environment were slightly shorter in Amsterdam than the native field site in Montana

### Controlled LD and SD freezing stress experiments

RIL and parental lines were grown at 20°C in growth chambers under LD and SD photoperiod regimes. The LD photoperiod was 14 hours light/10 hours darkness, while the SD photoperiod was 10 hours light/14 hours darkness. Details of Experimental Methods are given below, but are alluded to here in relation to plant growth. One-month-old plants were acclimated to cold by growing at 6°C for 3 weeks. A freezing treatment was done in darkness at -8°C for 24 hours. Ice crystallization was induced using ice chips. Plants and the soil were totally frozen. Plants were then returned to 6°C. Relative freezing tolerance in RILs and their parental lines was measured before and after 3 weeks of cold acclimation, and the electrolyte leakage was screened immediately after a freezing treatment of 24 hours, and 1 day after they were returned to 6°C to investigate their actual responses to selected freezing temperature. Maximum quantum yield of Photosystem II (Fv/Fm) was measured before and after cold acclimation, and 1 day after freezing treatment, and 1 day after they were returned to 6°C to observe the changes in the photosynthetic performance. To minimum a gradient in damage by harvesting samples, we conducted these experiments dividing into 6 sets, which consisted with 16~20 RILs per group. In addition, plants were randomly placed for the experiment.

### Outdoor environment (OE) experiment

The freezing stress experiment in Outdoor Environment (OE) was conducted in the winter and early spring of 2011–2012. Responses during and post freezing, and frost damage were evaluated in the same 108 RILs. Plants were germinated and transplanted in mid-October, 2011. Three weeks after transplanting, three individuals/line from 108 RILs plus 3 individuals of each parental line (n= 330 individuals total) were randomized in a research plot area at the University of Amsterdam, The Netherlands and were maintained until the

end of experiment. Figure 1 shows the minimum and maximum temperatures during the critical experimental period (January and February). It was a relatively warm early winter. The first sub-zero freezing event occurred on 16<sup>th</sup> January 2012 with a minimum temperature of  $-4^{\circ}\text{C}$ . In February there was a much colder and prolonged period of freezing. The minimum air temperature was  $-17^{\circ}\text{C}$  on 4<sup>th</sup> February 2012, the lowest temperature of the winter. Temperatures remained sub-zero until 13<sup>th</sup> February 2012. Responses of selected RILs and parental lines to outdoor freezing conditions were screened on both 17<sup>th</sup> January and 18<sup>th</sup> February 2012 by measuring Fv/Fm. Plants were frozen during the first and second cold spells, and were not covered with snow. Temperature was measured in the field with use of HOBO® data loggers for outdoor use. The logger was placed at plant height. Overall frost damage from the winter was scored in the middle of March 2012.

## Trait measurements

### Measurement of relative freezing tolerance before and after cold acclimation

—Relative plant freezing tolerance was assayed as described previously (Murray et al. 1989), with some modifications. Three uniform leaf disks per plant per line were cut (diameter size of 0.5cm) from non-acclimated or cold-acclimated plants with the use of a leaf disc borer and placed into test tubes containing 100 $\mu\text{l}$  de-ionized water. Test tubes were subsequently placed in a completely randomized design in a  $-1^{\circ}\text{C}$  Cooling Thermostats containing anti-freeze (Lauda Ecoline RE 312, Lauda Germany) for 1h after which ice crystals were added to nucleate freezing. After an additional 2h of equilibration at  $-1^{\circ}\text{C}$  the samples were gradually cooled in increments of  $-2^{\circ}\text{C}/\text{h}$ . A replicate sample from each genotype was removed every 2 h starting at  $-3^{\circ}\text{C}$  until the last samples were removed at  $-13^{\circ}\text{C}$ . Once removed the samples were stored on ice until all samples had been collected and left to thaw overnight in the cold room at  $4^{\circ}\text{C}$ . After thawing, all samples, including the unfrozen controls kept at  $4^{\circ}\text{C}$  (ELunfrozen), were incubated in 1ml de-ionized water with gentle shaking (125 motions/min) at room temperature for 2h. Electrolyte leakage from the leaves was measured using a conductivity meter (B-173 compact conductivity meter, Horiba Scientific). The samples were then placed for 1h in a  $-80^{\circ}\text{C}$  freezer, thawed for 30min in a  $57^{\circ}\text{C}$  stove, and shaken gently for an additional 2h before the conductivity of the resulting solution was measured to obtain a value for 100% electrolyte leakage (EL100). The percentage of electrolyte leakage from frozen leaves was calculated according to the equation as described by Webb et al. (1994):  $\%EL = (EL_{\text{frozen}} - EL_{\text{unfrozen}}) / (EL_{100} - EL_{\text{unfrozen}}) * 100$ . Response curves were developed for each plant and each  $LT_{50}$  was determined based on the sigmoidal response equation by the statistic program R (R Development Core Team. 2008). Two replicate measurements for each photoperiod/temperature combination were performed, and total 5280 individuals were used for this experiment (two temperature conditions  $\times$  two photoperiods  $\times$  six time samplings using 6 individuals  $\times$  two replicates  $\times$  108 RILs plus two parental lines ;  $n = 5280$  individual total).

### Measurement of maximum quantum yield of photosystem II (Fv/Fm)—Fv/Fm

was determined with a PAM-2000 chlorophyll fluorometer system (Heinz Walz, Effeltrich, Germany) using standard instrument settings (e.g. saturating pulse of 12,000  $\text{mmol m}^{-2} \text{s}^{-1}$  for 0.8s). Fluorescence data were recorded and computed with windows software for PAM fluorometers (2.133 version, Heinz Walz, Effeltrich, Germany). For determination of

Fv/Fm, one uniform leaf disc from middle part of the sixth leaf was removed and placed into black 96-well plates containing 200µl de-ionized water. Leaves were dark adapted for 20 min prior to determination of Fv/Fm. In dark-adapted plants,  $F_0$  and also levels of fluorescence measured at a very low photosynthetic photon flux density (PPFD) of,  $1 \text{ mmolm}^{-2} \text{ s}^{-1}$  and during a short light-saturating pulse (Fm) were measured and used to estimate the maximum quantum yield of PSII when fully oxidized ( $Fv/Fm = Fm - F_0/Fm$ ). Three replicate measurements were performed for measuring Fv/Fm, and total 2640 individuals were used for this experiment (four temperature conditions × two photoperiods × three replicates × 108 RIL plus two parental lines ; n= 2640 individual total).

**Measurement of electrolyte leakage during and post freezing**—Plant responses during and after freezing were measured by another type of electrolyte leakage assay (Scarpeci et al. 2008), with the protocol slightly modified. Three uniform leaf discs (Ø 0.5cm) from rosette leaves in the same plant being used for measurement of Fv/Fm were sampled with a leaf disc borer and immediately placed into 15ml Greiner tube containing 10ml de-ionized water. The discs were washed for 30min on a shaking platform to remove soil attached during cutting of the disc. Leaf discs were then placed in 12-well plates filled with 3ml of 0.01% Silwet 77 solution and maintained at 25°C for 1hr 30min on a shaking platform. Next, initial leakage was determined by measuring the electrical conductivity of the well plate solution, using a conductivity meter (B-173 compact conductivity meter, Horiba Scientific) with data expressed as  $\text{mS cm}^{-1}$ . The samples were then placed for  $2 \times 20$  sec in microwave (600W) and shaken gently for an additional 4 h before the conductivity of the resulting solution was measured to obtain a value for electrolyte leakage of the heat treated cells. Results were expressed as percentage of total conductivity ‘initial leakage / final leakage × 100’. Measurements were carried out in triplicate.

### Assessment of winter survival

Winter survival was visually scored on a scale of 0–10 using three individuals among families (0 : plant was killed, 10 : no apparent frost damage).

### Quantitative trait loci analysis

Single-trait, multi-trait linkage and mixed model multi-environment QTL analyses were performed with GenStat software (15<sup>th</sup> edition, VSN International, United Kingdom). To normalize results, all QTL analysis was carried out with log<sub>10</sub> transformed-adjusted means of each trait. For single-trait QTL analysis, the data was firstly used for the preliminary single environment analysis. Subsequently calculations of the genetic predictors were conducted with a step size of 2cM, and then an initial Genome Scan produced candidate QTL positions by Simple Interval Mapping (SIM). SIM results were then used as cofactors in a subsequent Genome Scan by Composite Interval Mapping (CIM), which was allowed to detect candidate QTLs. The final selection of significant QTL was obtained after a scan with backward elimination of putative QTL.  $LT_{50}$ , EL, and Fv/Fm results were subjected to a multi-trait linkage QTL analysis. To arrive at a multi-QTL model, the significant markers, or putative QTL, of the single marker analyses were used as the starting input set of predictor variables. After completion of this step, genome wide QTL scans by SIM and CIM were performed and then a multi-QTL model after backward selection was fitted to estimate QTL

locations and effects (Pastina et al. 2012). For Fv/Fm, mixed model multi-environment QTL analysis was additionally applied. The best suitable variance–covariance model was detected after environment exploratory analysis. Genetic predictors were computed for a step size of 2cM. The genetic model was built using the suggested candidate QTL as main or QTL × Environment (QTL×E) interactions. CIM was used for selecting the final QTL model. QTL significance levels and effects were determined by a final backward selection step at a significance level of 0.05. A significant QTL effect at particular genome positions was associated with a low P value, which was graphically shown on a  $-\log_{10}$  scale to resemble the typical LOD profile plot (Pastina et al. 2012). The additive effects, standard errors, high value alleles, the Percentage of Explained Variances (PVE) and positions of the QTLs were estimated and used to determine what traits were affected by the specific QTL.

### Statistical analysis

All further statistical tests were also performed using the GenStat software package (15<sup>th</sup> edition, VSN International, United Kingdom). To examine the correlation among traits measured across environmental conditions, correlation coefficients (Pearson's) were calculated. A principal component analysis (PCA) of Fv/Fm values under different conditions was also performed to visualize which environmental factors affected the Fv/Fm ratio of RILs most.

## RESULTS

### Relative freezing tolerance (RFT) under SD and LD conditions

*Boechea stricta* RILs and parental lines were grown under two photoperiods (SD and LD). The temperature at which 50% plants are killed by freezing ( $LT_{50}$ ) was estimated from fitted response curves. The average values, ranges and differences among RILs are presented in Table 1. Non-acclimated RILs and parental lines showed small differences in  $LT_{50}$  under either photoperiod conditions. After cold acclimation with SD grown plants, the  $LT_{50}$  of the parental lines LTM and SAD12 were  $-11.3^{\circ}\text{C}$  and  $-8.6^{\circ}\text{C}$ , respectively. Under LD growth conditions, the  $LT_{50}$  of the LTM and SAD12 were  $-10.4^{\circ}\text{C}$  and  $-8.1^{\circ}\text{C}$ , respectively (delta LTM to SAD =  $-2.3^{\circ}\text{C}$ ). In both conditions, there was significantly difference of freezing tolerance ( $p < 0.05$ ). LTM shows more freezing tolerance, but also more pronounced difference in SD relative to LD (delta of LTM SD-LD =  $-0.9^{\circ}\text{C}$ ; delta of SAD12 SD-LD =  $-0.5^{\circ}\text{C}$ ). In both light regimes, most RILs had intermediate  $LT_{50}$  values to the parents. Similar to results with parental lines, the LD grown RILs on average were less freezing tolerant than SD grown RILs.

### Electrolyte leakage (EL) and maximum quantum yield of photosystem II (Fv/Fm)

We quantified EL of leaves from plants exposed to freezing temperatures. EL increased during or after freezing treatment, and genotypic responses were highly associated with  $LT_{50}$  values. The maximum quantum yield of photosystem II, Fv/Fm, was measured to examine its relationship with cold acclimation ability and actual freezing tolerance. During cold acclimation, Fv/Fm decreased slightly in most of RILs. Although differences on Fv/Fm were detected among RILs, the variation in Fv/Fm was small and no significant correlation between  $LT_{50}$  and Fv/Fm was observed (Table S1). The freezing treatment in darkness for

24 h led to a significant reduction in Fv/Fm. Different responses of the Fv/Fm ratio and EL were observed among RILs and parental lines during and after freezing treatment (Table 1). Interestingly, Fv/Fm was highly correlated with electrolyte leakage assays (Table 2). Plants responded differently to freezing treatment, depending on the photoperiod during cold acclimation. Large variation among RILs was detected 1-day after freezing treatment among experimental time points in both photoperiod conditions (Table 1, see SD\_1DFT and LD\_1DFT). SAD12, which showed less freezing tolerance, also had lower Fv/Fm than LTM. Similarly, Fv/Fm was negatively affected by freezing, but the decrease of Fv/Fm in freezing tolerant RILs was much less than in freezing sensitive RILs (data not shown). These results indicate that components of the photosynthetic apparatus likely are damaged in freezing sensitive RILs, while freezing tolerant RILs were less affected.

### Maximum quantum yield and winter frost damage of outdoor environment (OE) freezing conditions

In Outdoor Environment (OE) conditions, we measured Fv/Fm to evaluate freezing injury after two freezing periods during the winter of 2011–2012 (Table 1). The first measurement was carried out during a mild freezing event (MF) in mid-January, the first freezing event of the year. Both parental lines had relatively low Fv/Fm values (LTM 0.66, SAD12 0.57), showing both lines suffered from the freezing event. The RILs showed a greater variation in values (0.41–0.72). The second measurement was performed after a prolonged severe freezing (SF) event in mid-February. Significant freezing damage was observed with Fv/Fm values for parental lines and RILs (LTM=0.38, SAD12=0.37, RILs=0.02–0.64). Thus, while the difference between the two parents was minimal in the severe cold period, more variation was observed in the RILs. Interestingly, genotypic responses to two outdoor freezing stress conditions did not correlate well with those under controlled freezing stress conditions (Fig. 2 and Table S1). The extreme freezing events of late winter caused the death of individual plants in some RILs. However, it was sometimes observed that overall winter damage scores for some RILs with low Fv/Fm had less final frost damage due to successful re-growth or recovery of the plants during warm temperatures in early spring.

### QTL analysis

Complex traits such as freezing tolerance need to be measured for collections of genotypes across multiple environments, because the relative performance of genotypes can change between environments, a well-known phenomenon called genotype by environment interaction. To identify constitutive and inducible QTLs controlling freezing tolerance in *B. stricta* efficiently, we performed multiple trait QTL analysis to test for pleiotropy or close linkage of QTLs, as well as mixed model based multi-environment single trait QTL analysis to detect QTLxEnvironment (QTLxE) interaction loci. Three QTLs were identified by traditional single trait analysis, i.e., on LG 3, 4 and 7 for Fv/Fm, EL and LT50, and the QTL of traits tended to be overlapping (Table 3). Multiple traits QTL analysis revealed that Fv/Fm was genetically associated with freezing tolerance-related traits. Multi-environment single trait QTL analysis using Fv/Fm identified three QTLs on LG 3, 4 and 7 again, QTL on LG 7 showed significant QTLxE interactions (Table 4).

### Single trait QTL analysis

For  $LT_{50}$  values of non-acclimated RILs in both LD and SD controlled conditions no significant QTLs were identified, likely due to the relative lack of trait variance among RILs. Two  $LT_{50}$  QTL regions for cold acclimated plants were identified, one that was SD specific (LG 3, marker A10) and one common to LD and SD (LG 4, marker At2g36390) (Fig. 3 and Table 3). For SD conditions, the accumulated total phenotypic variation explained by the two QTLs was 23.9% (11.7% by LG 3 and 12.2% by LG 4). The single QTL for  $LT_{50}$  was found only for long-photoperiod, with  $-\log_{10}(p) = 3.18$ , and phenotypic variation of this QTL was 11.0%. All superior alleles for freezing tolerance originated from the LTM parent from Montana, regardless of photoperiod.

For EL and Fv/Fm measurements from different photoperiods and experimental time points, CIM mapping identified a total of 11 QTLs. One putative QTL for Fv/Fm in acclimated plants grown under SD and LD was identified on linkage group 7. The QTLs on linkage group 7 explained 20.9% and 21.8% for short and long photoperiod, respectively. One QTL for Fv/Fm and EL was detected for the freezing treatment and the 1st day after freezing stress in both photoperiods, co-located on linkage group 4. We also identified other QTLs under different time points. One interesting QTL was identified on linkage group 3 for Fv/Fm at freezing treatment under short photoperiod, overlapping with the locus for  $LT_{50}$  under short photoperiod.

Under outdoor freezing stress conditions, no putative QTL was observed for overall winter damage. Fv/Fm measurements during winter, however, revealed one QTL region on linkage group 7 that exceeded the significance threshold across stress conditions.

### Multi-trait QTL analysis and multi-environment single trait QTL analysis

One QTL for EL collocated with a QTL for Fv/Fm from single trait QTL analysis under controlled environments. In order to confirm if freezing tolerant-related traits were associated with photosynthetic performance-related traits, a multi-trait QTL mapping approach was applied, in which variation for several traits was analyzed simultaneously. The results presented in Table S2 showed that most QTLs identified by single-trait analyses were still significant in the multi-trait QTL model. The QTL for EL and Fv/Fm detected on linkage group 4 post freezing was also identified in the multi-trait QTL analysis and the QTL for EL was strongly associated with Fv/Fm. This result suggests that these traits may be co-regulated. Multi-trait QTL analysis revealed exceptions for the short day/cold acclimated (SDCA) and short day/freezing treated (SDFT) conditions. The  $LT_{50}$  QTL on linkage group 3 in SDCA was no longer significant in multi-trait model, reflecting a lack of association with Fv/Fm. By contrast, multivariate analysis detected an additional QTL on linkage group 2 which was not identified with the single-QTL model for SDFT.

Multi-environment, single trait QTL analysis for Fv/Fm was performed to evaluate QTL by environment interaction. Three QTL  $\times$  E interaction loci were found, on linkage group 3, 4 and 7 (Fig. 4 and Table 4). The QTL of linkage group 3 was shown to be highly significant ( $-\log_{10}(p) = 6.57$ ), mainly due to a substantial effect from the specific environment (SDFT). Linkage group 4 contained a QTL ( $-\log_{10}(p) = 4.21$ ), which had relatively high effects on



Fv/Fm in controlled environments, but it had little effect in outdoor environments. The most significant QTL  $\times$  E effect ( $-\log_{10}(p) = 12.45$ ) in the multi-environment QTL model was detected on linkage group 7. Percentage of total phenotypic variation explained was between 0.2 and 22.3%, depending on environmental conditions. This locus had little effect on Fv/Fm in controlled non-acclimated and freezing treated conditions, but it was consistently present in cold or freezing stress conditions, with the superior LTM allele active in the most stressful conditions (Table 4). Our results suggest that QTLs on linkage group 7 would have important roles for freezing tolerance in all environments, while QTLs on linkage group 3 and 4 would be more environment-specific.

## DISCUSSION

*Boechera stricta* provides an excellent model system to study ecologically important traits such as freezing tolerance. There have often been observed differences in QTLs found under control versus outdoor conditions (Weinig et al. 2002; Martin et al. 2006). For example, a QTL study of flowering time in *B. stricta* identified different loci under different growth conditions (Anderson et al. 2011). Development of freezing tolerance can also be highly influenced by environmental factors such as temperature and photoperiod conditions. Thus, we conducted our QTL study under varying day-length conditions in a controlled environment, and also under outdoor conditions where plants experienced two periods of outdoor freezing stress to provide further information on freezing tolerance. We have found significant variation for freezing tolerance and photosynthetic performance to freezing stress conditions in *B. stricta*, and also show that photosynthetic performance-related trait may be genetically associated with freezing tolerance. Below, we discuss our findings and identify a potential candidate locus.

### Effect of photoperiod on freezing tolerance

Developing freezing tolerance is a complex phenomenon. Freezing tolerance is highly influenced by environmental factors such as duration of cold exposure, conditions of photoperiod, and developmental stage. Transcript profiling studies have shown that hundreds of genes are up-regulated in *A. thaliana* and they can be differently regulated with responses to environmental conditions in many species (Limin et al. 1997; Skinner et al. 2005; Vagujfalvi et al. 2005; Miller et al. 2006; Baga et al. 2007). Photoperiod condition is an important factors controlling freezing tolerance, but the genetic mechanism is still poorly understood.

In the climate chamber, we determined relative freezing tolerance (LT<sub>50</sub>) using one-month old RILs and their parents before and after cold acclimation for 3 weeks in different photoperiods. Electrolyte leakage (EL) was also quantified to assess the damage of cellular membranes during and after the freezing treatment. Our genetic analysis indicated that several regulatory factors may influence freezing tolerance in both photoperiods. A total of six LT<sub>50</sub>- and EL-related QTLs identified across experimental conditions showed intermediate effects. The most significant QTL for freezing tolerance was located on linkage group 4. This QTL was identified across experimental conditions (both LD and SD) and with two types of electrolyte leakage methods, indicating that it may contain genes

important for freezing tolerance by protecting membrane integrity. Another interesting QTL was identified on linkage group 3. Although the effect of this QTL was small, it was detected by LT50 measurements only after cold acclimation grown in the short photoperiod condition. This result indicates that short day can be contributed to the increase of freezing tolerance in *B. stricta*. It has been reported that freezing tolerance in *Gaura coccinea* is enhanced with cold treatment when they are exposed to short-day photoperiods (Pietsch et al. 2009). Similarly, cold acclimation ability in barley is enhanced by a short photoperiod condition in combination with low acclimating temperatures, which is associated with a short photoperiod induced delay in the transition from vegetative to reproductive stage (Flower et al. 2001). In *Arabidopsis*, two accessions also showed different levels of freezing tolerance in response to photoperiods, and phenotypic expression was traced to different QTLs (Alonso-Blanco et al. 2005). Although the role and mechanism for photoperiod on freezing tolerance in herbaceous species is unclear unlike woody plants (Li et al. 2003; Welling and Palva, 2006), this result suggests that that photoperiod conditions affecting freezing tolerance in *B. stricta* can also be under genetic control and lead to differential physiological, metabolic, and transcriptional adjustments during cold acclimation. The molecular mechanism and underlying gene(s) for the SD-specific QTL could not be elucidated in the current study.

Although remarkable progress has been made in understanding cold acclimation, the molecular mechanisms underlying how plants perceive low temperature and short photoperiod conditions during cold acclimation is still unclear. Further detailed study of the genetic variation present in *B. stricta* can uncover the adaptive mechanism for these differential freezing tolerances and photoperiod responses.

### **Close relationship between photosynthetic performance and freezing tolerance**

Low temperature stress can lead to a reduced rate of photosynthetic electron transport by strongly decreasing CO<sub>2</sub> fixation and increase relative excess energy, which can eventually lead to the production of potentially dangerous reactive oxygen species (ROS) and photo-inhibition of photosystem (Baker, 1994). Because production of ROS and occurrence of photo-inhibition may result in the disruptions of cold-induced physiological and biochemical changes necessary for cellular stability against freezing stress, overwintering species must increase their photosynthetic capacities to regulate such a balance in response to freezing condition during cold acclimation (Hüner et al. 1998). It has been demonstrated that the status of PSII is positively associated with freezing tolerance (Rizza et al. 2001; Pocock et al. 2001; Rapacz and Wolniczka, 2009). In our study, freezing tolerant and photoinhibition-related traits were evaluated, and QTL regions controlling these traits were compared. Like in previous studies, we found significant correlations between freezing tolerance and photoinhibition-related traits during freezing or post-freezing. Freezing treatments caused photoinhibition in the RILs, and the decrease in Fv/Fm was much higher in freezing sensitive RILs than in freezing tolerant RILs. These results indicated that components of PSII can be significantly damaged in freezing sensitive RILs. Results of our QTL analysis supported that these traits are mechanically or genetically associated, suggesting that key genes involved in the freezing tolerance can also control photo-inhibition related traits. This implies that a study of genes regulating reduced inhibition of photosynthesis in freezing

tolerant genotypes would provide new insights on the complexity of freezing tolerance in plants.

From a methodological point of view we found that chlorophyll fluorescence can be used as a reliable high-throughput phenotyping proxy for measuring freezing tolerance. It is a quick and inexpensive method widely used for analyzing the status of photosynthetic apparatus and understanding the mechanism by which a range of environmental factors alter photosynthetic activity (Baker, 2008). Among various chlorophyll fluorescence parameters, maximum quantum yield of photosystem II (Fv/Fm) has been applied for evaluating photosynthetic performance because it is the most easily measured and reflects a progressive inactivation of PSII-mediated electron transport. It was already suggested as one of promising methods to evaluate freezing tolerance of plants (Ehlert and Hinch, 2008), but few studies tested if it was a reliable high-throughput phenotyping method for measuring freezing tolerance. Chlorophyll fluorescence and electrolyte leakage methods were applied for our study. We studied the correlation between a direct method to measure freezing tolerance in plants and chlorophyll fluorescence. Our results demonstrated that there were significantly correlations between two methods. Routine frost damage assay such as electrolyte leakage is accurate, but laborious. Winter survival test is also relatively easy and can provide a reliable result, but experimental variability due to all kinds of uncontrolled factors accumulating in long term experiments can cause noisy result, and several years of replicated experiments are needed. They have disadvantages that are not easily applicable for large-scale screening purposes (Ehlert and Hinch, 2008). Our extensive comparative studies, therefore, support that chlorophyll fluorescence is a powerful tool for studying freezing tolerance in plants.

### Outdoor environment QTL and potential candidate gene

The genetic basis of research for understanding freezing tolerance is often obstructed by the difficulty in mimicking realistic outdoor environments under controlled conditions and in facing unpredictable environmental events or physiological damages in natural conditions. Significant and high correlations between Fv/Fm and two types of electrolyte leakage assessments were found during freezing and post freezing treatments in controlled environment conditions, indicating that Fv/Fm can be routinely used as a reliable phenotypic assessment for freezing tolerance in *B. stricta* for QTL study. Therefore, this approach was also applied for evaluating freezing tolerance in outdoor conditions. No QTL was detected for overall winter damage, which might be due to high levels of experimental noise under less controlled conditions outdoors. By contrast, data from Fv/Fm measured during winter identified one QTL. Our QTL analyses in more complex freezing conditions and different developmental stages thus identified one genomic region on linkage group 7 with strong effects on freezing tolerance in *B. stricta*.

Data analyses of Fv/Fm also provide the chance to study phenotypic plasticity of related factors. Although the QTL was not identified under controlled freezing conditions, the locus identified in nature environments also has relatively high and consistent effects in controlled stress conditions. This result suggests that it can be a form of phenotypic plasticity that is a key trait affecting fitness of *B. stricta* in variable environments. The locus at linkage group 7

is syntenic with a genomic region in *A. thaliana* that contains DREB1/CBF-type transcription factors. In *A. thaliana*, DREB1/CBF transcription factors have been suggested as a master regulator of morphological, physiological and biochemical adjustments for increasing freezing tolerance (Thomashow, 1999). They play an important role in the protection and stabilization of cellular membranes by inducing cryoprotectant solutes and cryoprotective proteins and, leading to improvement of anti-oxidative mechanisms in *A. thaliana* (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999). These genes are highly conserved in cold adaptable plants, and their importance for freezing tolerance has been reported in a number of species (Zhang et al. 2004; Skinner et al. 2005). Given that in *B. stricta* the LG 7 QTL shows consistent results in both controlled and outdoor conditions, this suggests that DREB1/CBF-type transcription factors may be promising candidates for unraveling molecular mechanism and fitness of freezing tolerance in this species.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

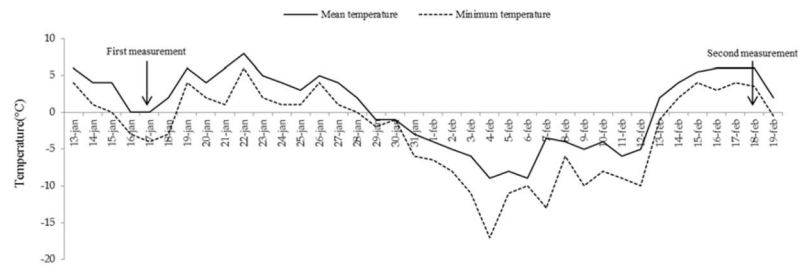
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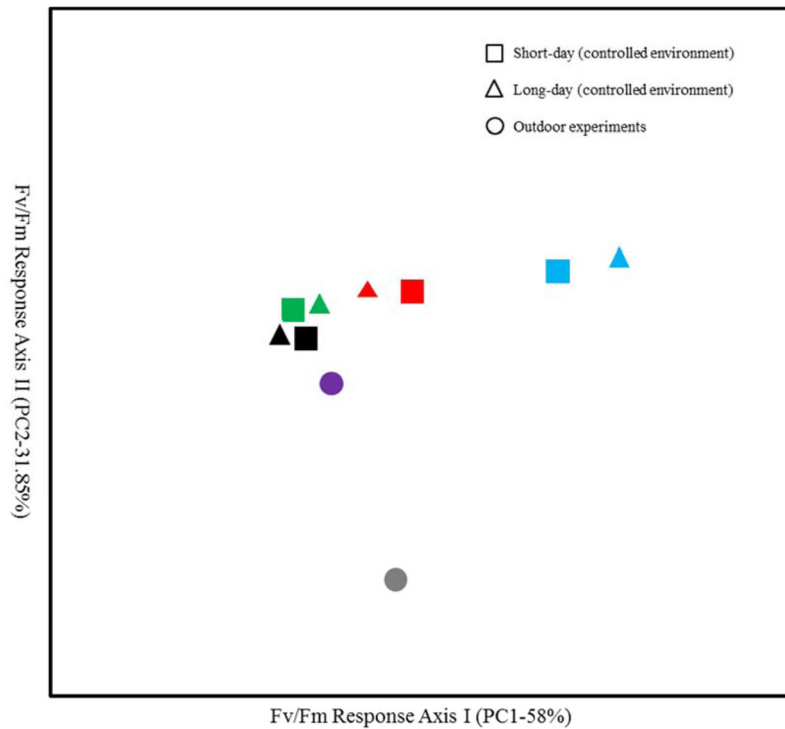
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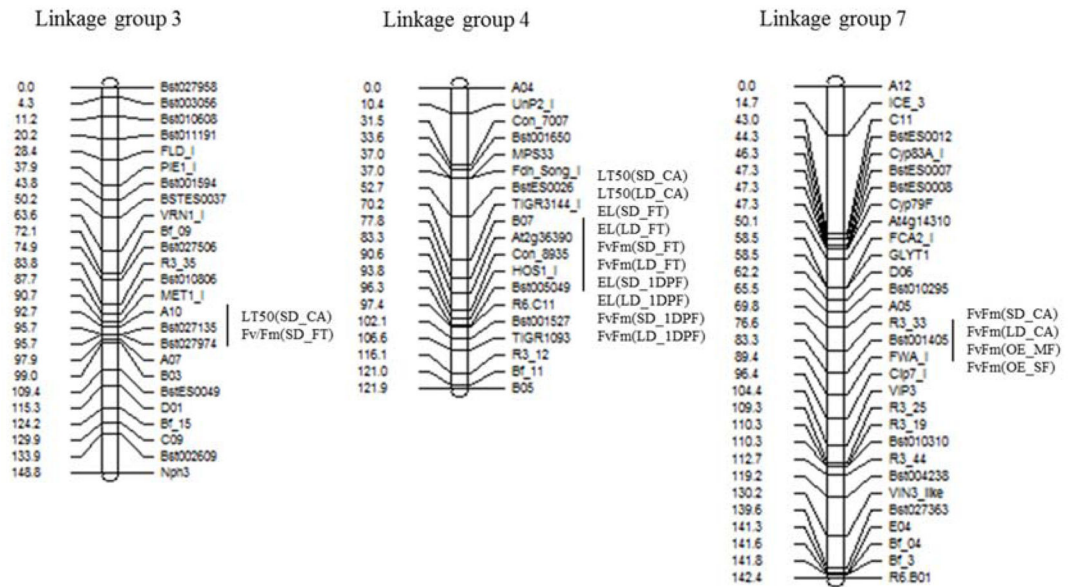


**Figure 1.** Maximum and Minimum daily temperatures for Outdoor Environment (OE) experienced by *Bochera stricta* Recombinant Inbred Lines (RILs) during January and February of 2012 in Amsterdam, The Netherlands. Responses to freezing-stress were assessed during a mild freezing event (January 18) and after a prolonged severe-freezing event (February 18). Three-week old plants were initially established outdoors in November 2011.

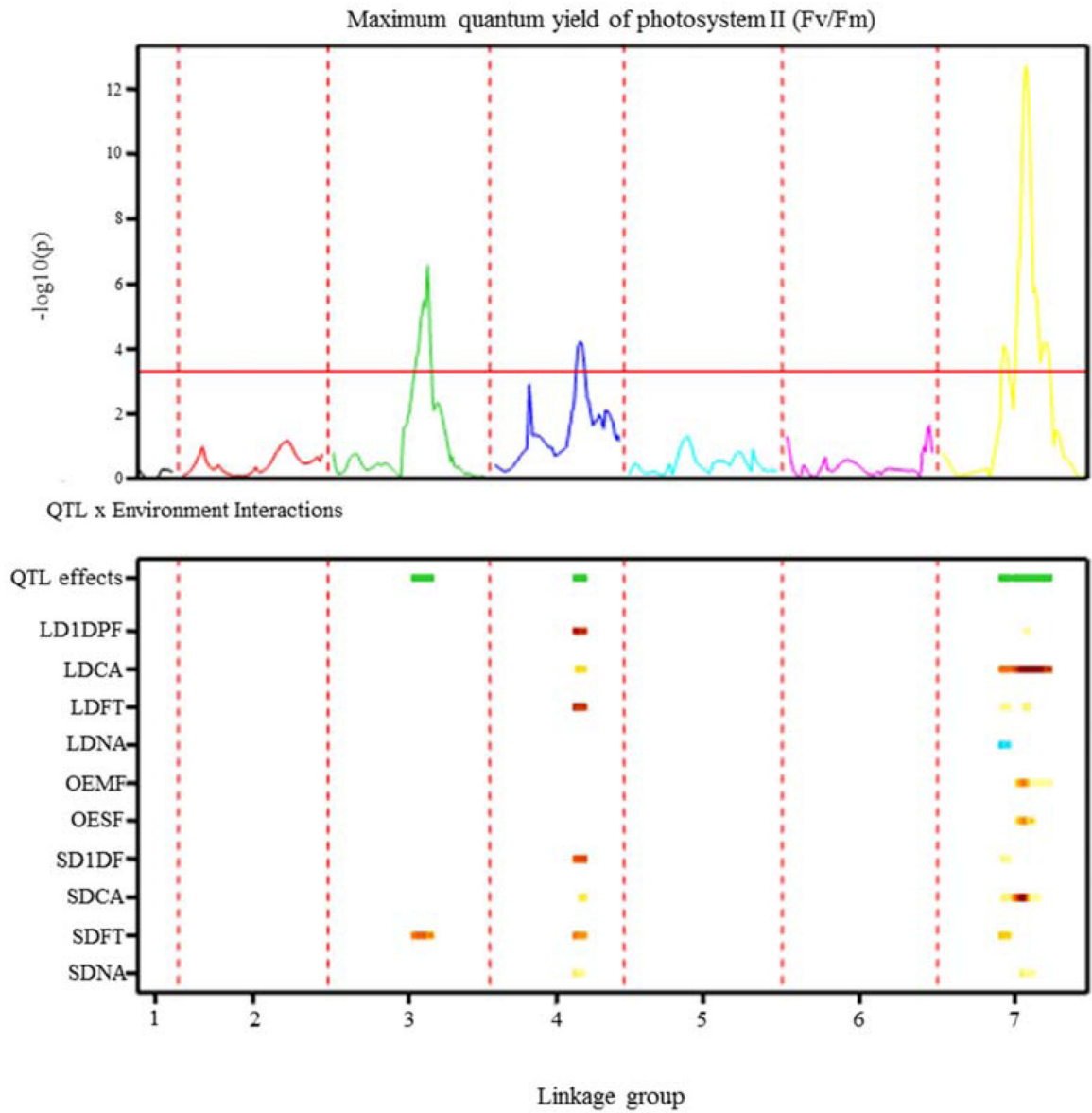


**Figure 2.** First two principal component axes of Fv/Fm responses of 108 Recombinant Inbred Lines of *Boechera stricta* grown under short-day/controlled environment (squares) long-day/controlled environment (triangles), and outdoor environment (circles) and measured after various temperature regimes (black = non-acclimated at 20°C; green = cold at 6°C for 3 weeks; red = freezing at -8°C for 24 hour;, blue = 1 day post-freezing; purple = outdoor mild-freezing January, 2012; gray = outdoor severe-freezing February, 2012).





**Figure 3.** Quantitative Trait Loci (QTL) identified for several traits under variable conditions on the three of the seven Linkage Groups (LG) of this study. SD; short day, LD ; long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, 1DF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012).



**Figure 4.** QTL profiles of LTM × SAD12 population, showing chromosomal regions with significant QTLs (upper panel) and their additive effect across environments. SD ; short day, LD ; long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, IDF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012).

**Table 1**  
 LT50, Fv/Fm and EL in the RIL population (LTM × SAD12) under control and stressed conditions

	LTM	SAD12	Range in RIL population	Mean in RIL population
LT50(SD_CA)	-11.10	-8.60	-5.10 ~ -14.20	-10.07
LT50(SD_NA)	-6.40	-5.70	-4.60 ~ -7.20	-6.21
Fv/Fm(SD_NA)	0.80	0.80	0.78 ~ 0.81	0.80
Fv/Fm(SD_CA)	0.77	0.77	0.75 ~ 0.79	0.77
Fv/Fm(SD_FT)	0.71	0.65	0.54 ~ 0.77	0.68
EL(SD_FT)	26.90	35.30	17.80 ~ 57.10	28.94
Fv/Fm(SD_IDPF)	0.71	0.58	0.21 ~ 0.77	0.63
EL(SD_IDPF)	24.80	44.60	16.20 ~ 74.10	32.69
LT50(LD_CA)	-10.40	-8.10	-4.70 ~ -14.20	-9.33
LT50(LD_NA)	-6.30	-5.80	-4.40 ~ -7.10	-6.00
Fv/Fm(LD_NA)	0.81	0.80	0.79 ~ 0.82	0.80
Fv/Fm(LD_CA)	0.77	0.76	0.74 ~ 0.78	0.76
Fv/Fm(LD_FT)	0.68	0.60	0.55 ~ 0.75	0.66
EL(LD_FT)	29.10	41.00	20.20 ~ 60.40	32.12
Fv/Fm(LD_IDPF)	0.67	0.42	0.16 ~ 0.77	0.57
EL(LD_IDPF)	28.80	52.50	18.20 ~ 80.10	39.00
Fv/Fm(OE_MF)	0.66	0.57	0.41 ~ 0.72	0.63
Fv/Fm(OE_SF)	0.38	0.37	0.02 ~ 0.64	0.38
OWFD	3.30	4.00	0.307,30	3.32

SD : short day, LD : long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, IDF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

**Table 2**  
Phenotypic correlations among traits under controlled environmental conditions

SD	LT50_CA	EL_FT	Fv/Fm_FT	EL_IDPF	FvFm_IDPF
LT50_CA	1				
EL_FT	.861**	1			
Fv/Fm_FT	-.912**	-.926**	1		
EL_IDPF	.874**	.910**	-.892**	1	
FvFm_IDPF	-.825**	-.867**	.829**	-.953**	1
LD	LT50_CA	EL_FT	Fv/Fm_FT	EL_IDPF	FvFm_IDPF
LT50_CA	1				
EL_FT	.805**	1			
Fv/Fm_FT	-.796**	-.836**	1		
EL_IDPF	.858**	.839**	-.804**	1	
FvFm_IDPF	-.809**	-.804**	.780**	-.969**	1

\*\* means correlation is significant at the 0.01 level (2-tailed) and \* means correlation is significant at the 0.05 level (2-tailed).

SD ; Short-day, LD ; Long-day, CA ; cold acclimation, FT ; freezing treatment, IDF : 1 day post freezing treatment

Summary of putative QTLs controlling freezing tolerant and photosynthetic performance related traits in the single trait QTL model

**Table 3**

Condition	Trait	Linkage group	Locus name	-log <sub>10</sub> (p)	% Expl. Var.	Add.eff	High value allele
	LT50_SD	3	A10	3.95	11.69	0.03	LTM
	LT50_SD	4	A2g36390	3.94	12.21	0.03	LTM
Cold acclimation	LT50_LD	4	A2g36390	3.17	11.01	0.02	LTM
	Fv/Fm_SD	7	Bst001405	5.42	20.95	0.00	LTM
	Fv/Fm_LD	7	Bst001405	5.63	21.76	0.00	LTM
<hr/>							
	EL_SD	4	A2g36390	4.01	14.21	0.04	SAD12
	EL_LD	4	A2g36390	3.12	10.83	0.03	SAD12
Freezing treatment	Fv/Fm_SD	3	A10	4.48	13.86	0.01	LTM
	Fv/Fm_LD	4	A2g36390	3.01	8.89	0.01	LTM
	Fv/Fm_LD	4	A2g36390	3.97	14.07	0.01	LTM
<hr/>							
1 day post freezing treatment	EL_SD	4	A2g36390	4.90	17.52	0.07	SAD12
	EL_LD	4	A2g36390	5.07	18.16	0.07	SAD12
	Fv/Fm_SD	4	A2g36390	3.48	12.18	0.03	LTM
	Fv/Fm_LD	4	A2g36390	3.93	13.91	0.05	LTM
<hr/>							
Outdoor environment	Fv/Fm_MF	7	Bst001405	3.95	15.06	0.01	LTM
	Fv/Fm_SF	7	Bst001405	3.98	15.18	0.09	LTM

SD ; short day, LD ; long day, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

**Table 4**

Descriptive summary results from multi-environment QTL model for Fv/Fm

Linkage group	Locus name	Position	$-\log_{10}(p)$	Environment	Add.eff.	High value allele	% Expl. Var.	P	s.e.
3	A10	92.74	6.57	SD_NA	0.00	SAD12	0.10	0.74	0.00
				LD_NA	0.00	SAD12	0.00	0.90	0.00
				SD_CA	0.00	LTM	0.00	0.82	0.00
				LD_CA	0.00	SAD12	0.00	0.93	0.00
				SD_FT	0.02	LTM	12.80	0.00	0.01
				LD_FT	0.00	LTM	0.00	0.98	0.00
				SD_IDPF	0.02	LTM	2.10	0.11	0.01
				LD_IDPF	0.02	LTM	1.00	0.26	0.01
				OE_MF	0.01	LTM	0.60	0.39	0.01
				OE_SF	0.01	LTM	0.90	0.32	0.01
4	At2g36390	83.33	4.21	SD_NA	0.00	LTM	5.00	0.02	0.00
				LD_NA	0.00	LTM	0.00	0.89	0.00
				SD_CA	0.00	LTM	2.60	0.08	0.00
				LD_CA	0.00	LTM	5.40	0.01	0.00
				SD_FT	0.02	LTM	9.40	0.00	0.01
				LD_FT	0.02	LTM	15.20	0.00	0.01
				SD_IDPF	0.04	LTM	13.40	0.00	0.01
				LD_IDPF	0.06	LTM	17.20	0.00	0.01
				OE_MF	0.00	LTM	0.10	0.70	0.01
				OE_SF	0.01	SAD12	0.50	0.45	0.01
7	Bst001405	83.33	12.45	SD_NA	0.00	LTM	4.60	0.04	0.00
				LD_NA	0.00	SAD12	0.20	0.65	0.00
				SD_CA	0.00	LTM	21.40	0.00	0.00
				LD_CA	0.00	LTM	22.30	0.00	0.00
				SD_FT	0.01	LTM	2.50	0.08	0.01
				LD_FT	0.01	LTM	4.40	0.03	0.01
				SD_IDPF	0.02	LTM	3.00	0.07	0.01

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Linkage group	Locus name	Position	$-\log_{10}(p)$	Environment	Add.eff.	High value allele	% Expl. Var.	P	s.e.
				LD_IDPF	0.03	LTM	3.60	0.04	0.01
				OE_MF	0.02	LTM	13.90	0.00	0.01
				OE_SF	0.05	LTM	12.90	0.00	0.01

SD : short day, LD : long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, IDF : 1 day post freezing treatment, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)