ORIGINAL PAPER

A novel major quantitative trait locus controlling seed development at low temperature in soybean (*Glycine max*)

Tatsuya Ikeda · Shizen Ohnishi · Mineo Senda · Tomoaki Miyoshi · Masao Ishimoto · Keisuke Kitamura · Hideyuki Funatsuki

Abstract Low temperature is among the critical environmental factors that limit soybean production. To elucidate the genetic basis for chilling tolerance and identify useful markers, we conducted quantitative trait loci (QTL) analysis of seed-yielding ability at low temperature in soybean (*Glycine max*), using artificial climatic environments at usual and low temperatures and recombinant inbred lines derived from a cross between two contrasting cultivars in terms of chilling tolerance. We identified a QTL of a large effect (LOD > 15, $r^2 > 0.3$) associated with seed-yielding ability only at low temperature. The QTL was mapped near marker Sat_162 on linkage group A2, where no QTL for chilling tolerance has previously been identified. The tolerant genotype did not increase the pod number but maintained the seed number per pod and single seed weight, namely, the efficiency of seed development at low temperature. The effect of the QTL was confirmed in a segregating population of heterogeneous inbred families, which provided near-isogenic lines. The genomic region containing the QTL also influenced the node and pod numbers regardless of temperature condition, although this effect was not primarily associated with chilling tolerance. These results suggest the presence of a new major genetic factor that controls seed development specifically at low temperature. The findings will be useful for marker-assisted selection as well as for understanding of the mechanism underlying chilling tolerance in reproductive organs.

Communicated by H. T. Nguyen.

T. Ikeda · K. Kitamura
Department of Plant Genetic Resources,
Graduate School of Agriculture, Hokkaido University,
9 Kita 9 Nishi, Sapporo, Hokkaido 060-8589, Japan

S. Ohnishi · T. Miyoshi
Tokachi Agricultural Experiment Station,
Shinnsei, Memuro, Hokkaido 082-0071, Japan

Present Address:
S. Ohnishi
Hokkaido Central Agricultural Experiment Station,
Naganuma, Hokkaido 069-1395, Japan

M. Senda
Faculty of Agriculture and Life Science, Hirosaki University,
Bunkyo-cho 3, Hirosaki, Aomori 036-8561, Japan

M. Ishimoto · H. Funatsuki (✉)
National Agricultural Research Center for Hokkaido Region,
Hitsujigaoka, Toyohira-Ku, Sapporo, Hokkaido 062-8555, Japan
e-mail: funazki@affrc.go.jp

Introduction

Low temperature is among the critical environmental factors that limit agricultural production worldwide. Therefore, a large number of studies have dealt with low-temperature stress in plants with the final goal of improving chilling/freezing tolerance (Mahajan and Tuteja 2005). Extensive studies at the molecular level have advanced our understanding of the regulatory mechanisms underlying response and tolerance to low temperature, (for review, see Thomashow 1999; Yamaguchi-Shinozaki and Shinozaki 2006; Chinnusamy et al. 2007; Penfield 2008). Quantitative trait loci (QTL) analysis has also been conducted to unravel the genetic basis for low-temperature tolerance and identify molecular markers useful for marker-assisted selection (MAS) (e.g., Andaya and Mackill 2003a; Jompuk et al. 2005). However, plant materials used in those works were biased toward vegetative organs. As a next step, molecular

genetic analysis on chilling tolerance in reproductive organs of crop plants is important to identify key regulators of stable seed-yielding ability under stress conditions since the regulatory mechanisms might differ in different tissues (Chinnusamy et al. 2007). It should also be noted that some studies showed no correlation between tolerance to stress in vegetative organs and seed productivity (Leipner et al. 2008), which is the final target in many crops.

Some varieties of soybean (Glycine max) in Hokkaido, the northernmost island of Japan, are more tolerant to low temperatures at the reproductive phase than are standard varieties (Raper and Kramer 1987). QTL analysis has been conducted to identify loci associated with such chilling tolerances (Funatsuki et al. 2005). A major QTL was identified in the vicinity of the T locus, which had previously been found to be associated with chilling tolerance with the T allele conferring a higher level of chilling tolerance (Morrison et al. 1994; Takahashi and Asanuma 1996). The QTL is likely to affect every yield component (Funatsuki et al. 2005) as well as vegetative growth at low temperatures (Kurosaki et al. 2004). In addition, some maturity genes, which control flowering and maturity times (Palmer et al. 2004), have been reported to be associated with chilling tolerance (Kurosaki et al. 2004; Funatsuki et al. 2005; Takahashi et al. 2005). The E1e3e4 alleles at the maturity loci conferred higher levels of chilling tolerance than did other genotypes. However, little is known about other QTLs for chilling tolerance although several cultivars without the T and E1e3e4 alleles are tolerant to low temperatures at the reproductive phase.

In the present study, we conducted QTL analysis of soybean chilling tolerance at the reproductive phase using recombinant inbred lines (RILs) derived from a cross between a chilling-sensitive variety and a chilling-tolerant variety without the T and E1e3e4 alleles (Shirai et al. 2004; H. Funatsuki, unpublished results). The objectives of this study were: (1) to identify new QTL for chilling tolerance, (2) to characterize the effects of the QTL on yield components and (3) to clarify the genetic association between the QTL and other traits.

Materials and methods

Plant materials

A total of 192 RILs at the F_5 and F_6 generations were derived from the cross between the soybean (Glycine max (L.) Merr.) varieties Toyomusume (TM) and Toyoharuka (TR). TR is among the most chilling-tolerant varieties without the T allele (Shirai et al. 2004), while TM is relatively chilling-sensitive (Kurosaki and Yumoto 2003). F_6 RILs were derived from the F_5 plants grown in growth

chamber D, which is described below. Heterogeneous inbred families (HIFs) segregating for the marker closest to the putative QTL for chilling tolerance were developed as described below and were used.

Plant growth conditions and phenotyping of RILs

A total of 156 RILs were grown in growth chambers to estimate chilling tolerance for seed yield according to the method developed by Funatsuki et al. (2005) with several modifications. The F_5 and F_6 plants were grown in 2006 and 2007, respectively. Seeds were sown in March of both years. Two plants of a RIL in each generation were used to evaluate chilling tolerance of the RIL. One plant was subjected to chilling treatment, while the other plant was used as a control. Since one pot contained two plants, plants from two RILs were grown in a pot. The combinations of the RILs were decided randomly for the F_5 generation, while RILs with similar flowering and maturing times were grown in the same pot for the F_6 generation. Parental cultivars were planted in three pots in each temperature regime in each year. A set of 84 pots were placed in each environment in each year: 78 pots for RILs and 6 for the parental varieties.

The temperature regimes were set according to Method III described by Funatsuki et al. (2004). Plants were grown until first flowering at 22/17°C with 15 h light in walk-in growth chambers A and B. One of a pair of pots was placed in growth chamber A and the other was placed in growth chamber B. The pots were placed randomly, but the paired pots were set at the same position in each chamber. To minimize the effect of possible heterogeneous climate within these growth chambers, the positions of the pots were changed within the chambers every week. Upon flowering of plants, the pots in growth chamber A were moved to the chilling environment (15/15°C) and those in growth chamber B were transferred to the control environment (24/17°C). For different environments, another two walk-in growth chambers (C and D) were used. Growth chambers C and D were used for chilling and control environments, respectively. The placement of pots and the rotation were carried out in the same way in growth chambers C and D.

Plant height, flowering and maturity times, node number, pod number, seed number, seed weight and pod wall weight were recorded for each plant. Flowering and maturity dates were scored at first flowering and at discoloration of 95% pods corresponding to the developmental stages of R1 and R8 (Fehr et al. 1971), respectively. Seeds of less than 2 mm in diameter at maturity were excluded. Seed weight was calibrated to contain 15% moisture content using a moisture meter (PM-830-2, Kett Science, Tokyo, Japan), while pod wall weight was scored as DW.

Genotyping of RILs, HIFs and seeds

The F_5 RIL plants (156 RILs grown in growth chambers C and D and an additional 36 RILs grown in a greenhouse) were used for genotyping. DNA was isolated from leaves or seeds as described by Funatsuki et al. (2008).

Polymerase chain reactions (PCR) for simple sequence repeat (SSR) markers were performed as described previously (Funatsuki et al. 2005) with a few modifications of the use of a 384-well thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA) in addition to a 96-well thermal cycler and the use of AmpliTaq Gold polymerase (Applied Biosystems).

PCR products were separated using 3.5% (w/v) agarose, 6% (w/v) non-denaturing polyacrylamide and 6% (w/v) denaturing polyacrylamide gel electrophoreses according to the size difference between amplicons for the parental cultivars as described previously (Funatsuki et al. 2005).

Map construction

TM and TR were tested with 714 simple sequence repeat (SSR) markers previously placed on integrated soybean genetic linkage maps (Yamanaka et al. 2001; Song et al. 2004). In the regions where markers were placed densely and polymorphic markers had previously been found, no more markers were screened. One hundred and five polymorphic markers were chosen to avoid redundancy in terms of map position. The 192 RILs were genotyped with these SSR markers. The F_5 RIL plants grown in growth chamber C were genotyped with SSR markers on the linkage groups (LGs) where QTL were suggestively found using the map constructed for plants in growth chamber D. MAPMAKER/ EXP 3.0b (Lander et al. 1987) was used to determine the LGs and the orders of the markers with the threshold of minimum logarithm of odds (LOD) value of 3.0. Map-Manager QTX (Manly et al. 2001) was used to estimate genetic distances between the markers. When unlinked LGs and markers were located on the same linkage group of the map of Song et al. (2004) and MapManager QTX confirmed the linkage between the LGs/markers ($P < 0.05$), these markers were placed on the same linkage group with the order determined by Song et al. (2004). The heterozygotes at each marker locus were dropped from the data set according to the programs used. The analysis employed the default values including Haldane map function. The nomenclature of LGs was according to Song et al. (2004), based on the common markers mapped.

Production and growth conditions of HIFs

A HIF, which is also known as the next generation of a residual heterozygous line (Yamanaka et al. 2005), is developed from an RIL in which the genomic region of interest is segregated with the other regions being fixed and can provide plant materials equivalent to near-isogenic lines (Tuinstra et al. 1997). HIFs were developed from RILs that were heterozygous at Sat_162 (forward primer 5′-GCG TGG TTT TTC GCT GGA TAT A-3′, reverse primer 5′-GCG CAT TTC GTA ACA TAT TTT TCA C-3′), the nearest marker to the putative major QTL for chilling tolerance. The F_6 seeds in the F_5 plants grown in growth chamber D were genotyped at Sat_162 and were sorted into TR, heterozygous and TM genotypes. Thus, the progeny derived from an F_5 plant constituted an HIF.

Seeds of 11 HIFs were sown in August 2007. Two pots for each homozygous genotype of each HIF were placed in growth chamber A with one pot containing two plants. Upon flowering, one of the two pots with the same genotype was moved to growth chamber C and the other was moved to growth chamber D. Consequently, a total of 22 plants with the same genotype at Sat_162 were examined for a temperature regime. The other growth conditions were identical to those for the RILs except for the growing season.

Data analysis

Since it is useful to dissect a complex trait into component traits for QTL analysis (Yin et al. 2004), total seed weight per plant, which is equivalent to seed yield, was resolved into its components, pod number per plant, seed number per pod and single seed weight as follows:

$$\text{Total seed weight} = (\text{Pod number per plant}) \times (\text{seed number per pod}) \times (\text{single seed weight}).$$

Normality test with the Shapiro-Wilk index (Shapiro and Wilk 1965), analysis of variance (ANOVA), and correlation and regression analyses were carried out using the PROC UNIVARIATE, GLM, CORR and REG procedures, respectively (SAS Institute 1996). The PROC GLM procedure was also used to calculate the r^2 value of the marker class in one-way ANOVA. Comparison between the genotypes at Sat_162 in HIFs was performed by using the pairwise t test with the mean values for the lines of the TM and TR genotypes in the same family being a pair.

QTL analysis

QTL analysis was performed using the phenotypic trait values of the RILs and the linkage maps constructed as described above. The F_6 generation was analyzed with the genotypes of corresponding parental F_5 plants since the heterozygous genotype at any marker was ignored in the programs used. Single marker regression analysis was

performed using MapManager QTX. Composite interval mapping (CIM) was performed using QTL Cartographer ver. 2.5 (Wang et al. 2005). We ran CIM model 6 with a window size of 10 cM for each trait separately. Marker covariates for CIM were identified using a stepwise forward and backward regression ($P = 0.1$). The genome was scanned at 2-cM intervals. Confidence intervals for QTL position were established as the region around the peak location where there is one order of magnitude (1 LOD) change in the probability of a false-positive (Lander and Botstein 1989). One thousand permutation tests were performed on each trait to establish empirical LOD thresholds at the 5% α level for experiment-wise Type I error (Churchill and Doerge 1994).

Results

Phenotypes of parents

All of the seed yield components of the chilling-sensitive variety Toyomusume (TM) decreased in the chilling environment, resulting in a drastic reduction of total seed weight per plant (Table 1). In TM, some pods with normally developed pod walls contained no visible seeds (Fig. 1a, c). On the other hand, the chilling-tolerant variety Toyoharuka (TR) maintained seed number per pod and single seed weight at high levels in the chilling environment, while the pod number per plant decreased similarly to that in TM (Table 1, Fig. 1b, c). Total seed weight per plant in the chilling environment was consequently much

larger in TR than in TM. TR had a plant architecture of fewer nodes on branches, in agreement with the fact that this variety is recognized as a low-branching type (Shirai et al. 2004). Accordingly, TM had larger numbers of pods in both environments than did TR. Regarding the traits of the main stem, the two varieties were very similar in the control environment, while large differences in seed number per pod and single seed weight were found in the chilling environment, indicating that the greater decrease in seed-yielding ability of TM in the chilling environment is not attributed simply to the nature of TM having more pods on branches, a large part of which developed after the exposure to low temperature. These results indicated that alleviation of the drastic drop in seed number per pod and single seed weight at low temperature is a major factor for chilling tolerance of TR.

Phenotypic variation among RILs

Phenotypic variation for yield components among the F_6 RILs is shown in Fig. 2. While the frequency distributions of the RIL population for seed number per pod and single seed weight in the control environment fitted normality (Fig. 2c, e), those in the chilling environment significantly deviated from normality (Fig. 2d, f), especially due to the low values of kurtosis (-0.67 for seed number per pod and -1.18 for single seed weight). These results suggested the presence of major genetic factors controlling seed number per pod and single seed weight specifically in the chilling environment. While the frequency distributions for pod number and total seed weight per plant were distorted

Table 1 Mean values (±standard error, $n = 6$) for the traits measured in the two parental soybean varieties at control and low temperatures

Trait	Control temperature		Low temperature		CTI[a]	
	TM	TR	TM	TR	TM	TR
Whole plant						
No. of nodes	22.3 ± 0.9	14.5 ± 1.7**[b]	19.0 ± 0.5	14.2 ± 2.4*	85	98
Total seed weight (g)	27.9 ± 1.3	18.9 ± 0.9***	1.9 ± 0.4	6.5 ± 0.3****	7	34
No. of pods	44.7 ± 0.9	26.2 ± 1.6****	21.5 ± 1.2	11.8 ± 0.7****	48	45
No. of seeds per pods	1.53 ± 0.08	1.62 ± 0.14 ns	0.74 ± 0.07	1.50 ± 0.05****	49	92
Single seed weight (mg)	408 ± 9	458 ± 10**	118 ± 11	371 ± 13****	29	81
Main stem						
No. of nodes	10.0 ± 0.3	10.0 ± 0.0 ns	9.8 ± 0.2	10.2 ± 0.3 ns	98	102
Total seed weight (g)	16.0 ± 1.4	16.4 ± 1.2 ns	1.4 ± 0.2	6.1 ± 0.4 ****	9	37
No. of pods	23.7 ± 1.7	20.3 ± 1.4 ns	13.7 ± 0.7	10.8 ± 0.9 *	58	53
No. of seeds per pods	1.59 ± 0.05	1.75 ± 0.09 ns	0.84 ± 0.04	1.54 ± 0.08 ****	53	88
Single seed weight (mg)	428 ± 13	465 ± 12 ns	119 ± 12	374 ± 15 ****	28	80

TM var. Toyomusume, *TR* var. Toyoharuka

[a] $CTI = \dfrac{\text{(Mean trait value at the low temperature)}}{\text{(Mean trait value at the control temperature)}} \times 100$

[b] Significant at the levels of 0.05*, 0.01**, 0.001*** and 0.0001**** by Student's t test

Fig. 1 Pods of soybeans grown in the chilling environment at maturity. Plants were grown at 15°C from the stage of flowering to maturity. **a** Pods on the uppermost node of a main stem of chilling-sensitive var. Toyomusume(*TM*). **b** Pods on the uppermost node of a main stem of chilling-tolerant var. Toyoharuka (*TR*). **c** Insides of the pods shown in **a** (*left*) and shown in **b** (*right*). **d** *Opened pods of lines* of the TM and TR genotypes at Sat_162 from a heterogeneous inbred family. Pods in a row were derived from a single plant
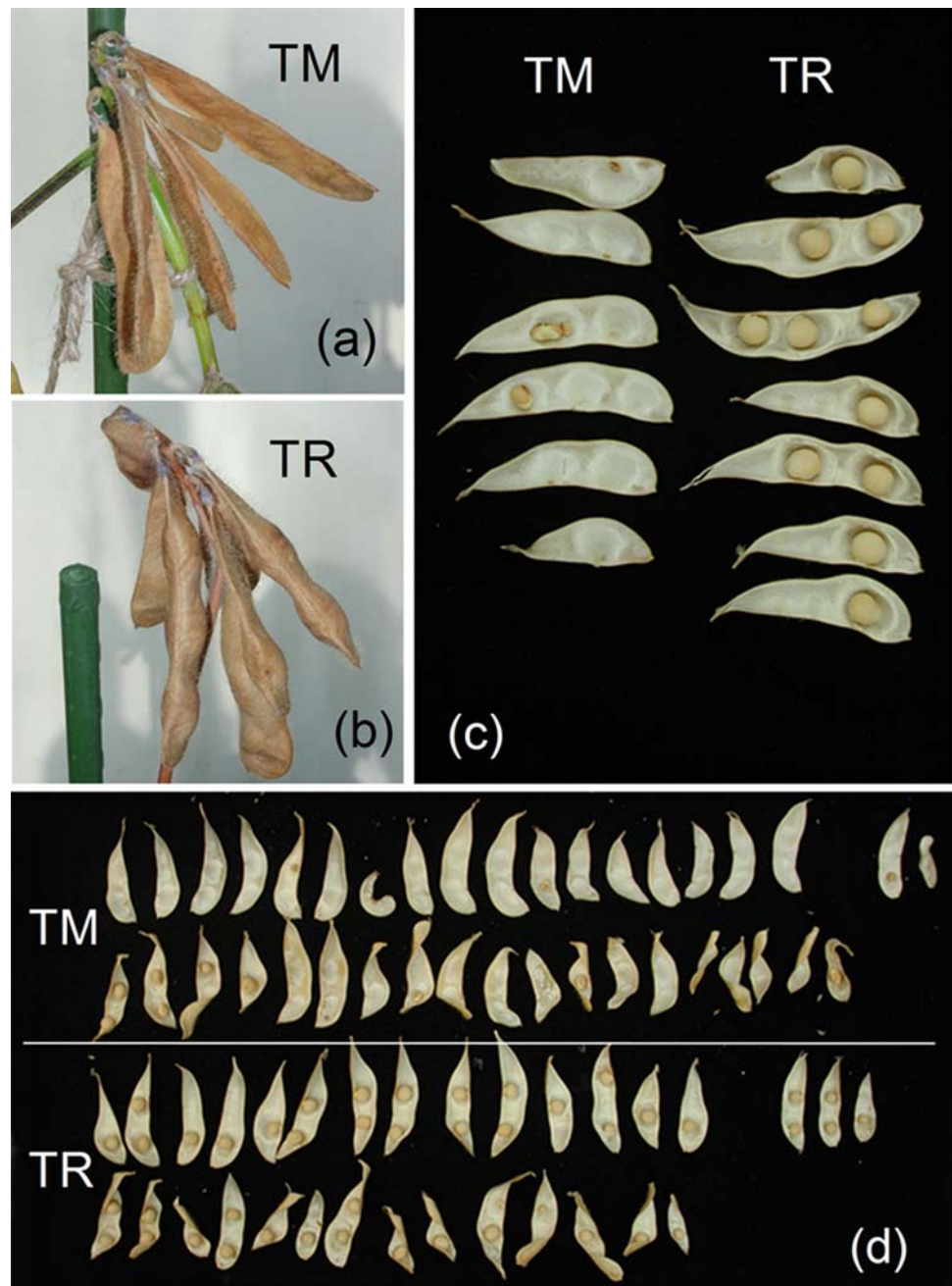
regardless of the environment (Fig. 2a, b, g, h), a lower value of kurtosis was scored for total seed weight in the chilling environment compared with that for total seed weight in the control environment.

QTL analysis of chilling tolerance, plant architecture and maturing habit

The resulting genetic linkage map covered 1,508 cM (Table 2). Few or no polymorphic markers were found in some regions, resulting in the separation of several of 20 linkage groups (LGs) constructed by Song et al. (2004).

Consequently, the markers constituted 25 LGs and seven unlinked markers.

QTL analysis of yield components and several traits associated with plant architecture and maturing habit was conducted for the F_5 and F_6 RILs grown in the control and chilling environments using the linkage map constructed. Single marker regression analysis was first performed for the unlinked markers, resulting in no significant association of these markers with the traits examined. CIM at the empirical threshold values for seed number per pod and single seed weight identified a highly significant QTL near simple sequence repeat (SSR) marker Sat_162 on LG A2
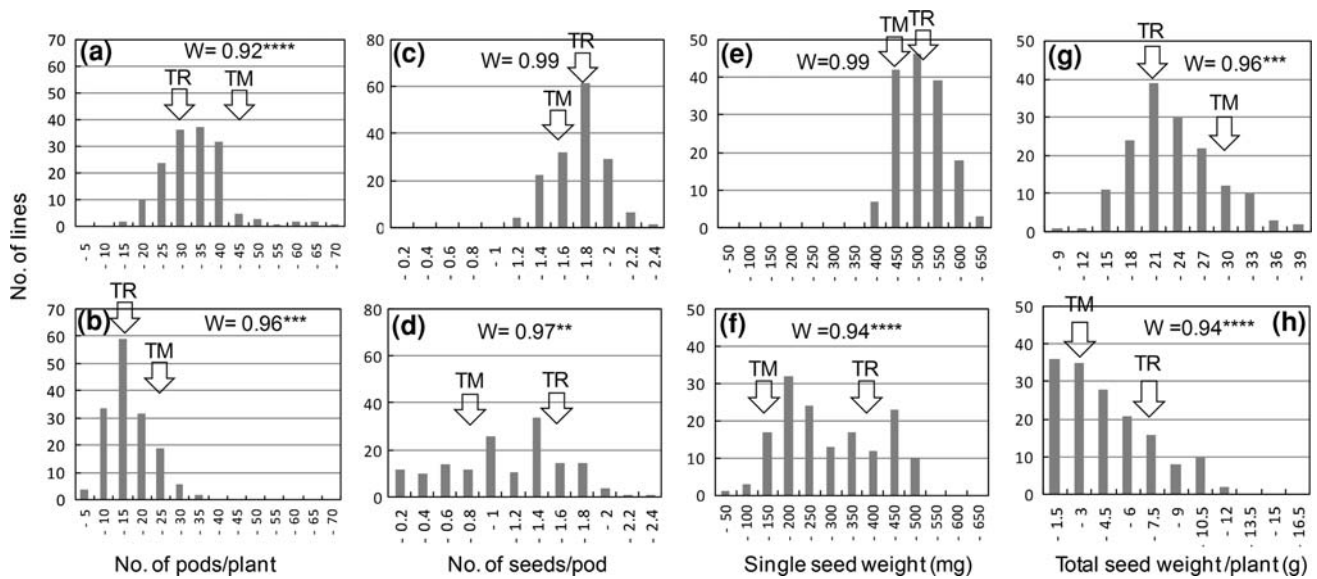
Fig. 2 Frequency distributions of F_6 recombinant inbred lines (RILs) for traits associated with seed-yielding ability. Traits are pod number per plant (**a**, **b**), seed number per pod (**c**, **d**), single seed weight (**e**, **f**) and total seed weight per plant (**g**, **h**) at control (**a**, **c**, **e**, **g**) and low (**b**, **d**, **f**, **h**) temperatures. The vertical axes represent number of RILs, while the horizontal ones indicate trait values. Parental mean values are indicated with arrows (TM chilling-sensitive var. Toyomusume, TR chilling-tolerant var. Toyoharuka). W indicates Shapiro-Wilk index. Deviation from normality was significant at the levels of 0.01**, 0.001*** and 0.001****

Table 2 Markers used and linkage groups

LG in IM[a]	No. of markers					No. of LGs	Map length (cM)
	Screened	Polymorphic	Mapped	Unlinked	Linked		
A1	41	3	2	0	2	1	17
A2[b]	46	8	7	0	7	1	166
B1	29	8	4	1	3	1	64
B2	16	14	7	0	7	1	75
C1	32	6	4	1	3	1	41
C2	48	4	4	1	3	1	69
D1a	36	11	5	0	5	2	66
D1b	46	11	7	0	7	1	143
D2	47	10	6	1	5	2	67
E	42	5	3	1	2	1	8
F	36	14	8	1	7	1	133
G	29	11	5	0	5	1	64
H	31	8	6	0	6	1	148
I	32	5	3	1	2	1	12
J	47	5	3	0	3	1	0
K	24	5	4	0	4	1	69
L	31	9	9	0	9	2	116
M	45	8	5	0	5	2	74
N	25	15	6	0	6	1	77
O	31	17	7	0	7	2	98
Total	714	177	105	7	98	25	1,508

[a] Linkage groups on the integrated map constructed by Song et al. (2004)

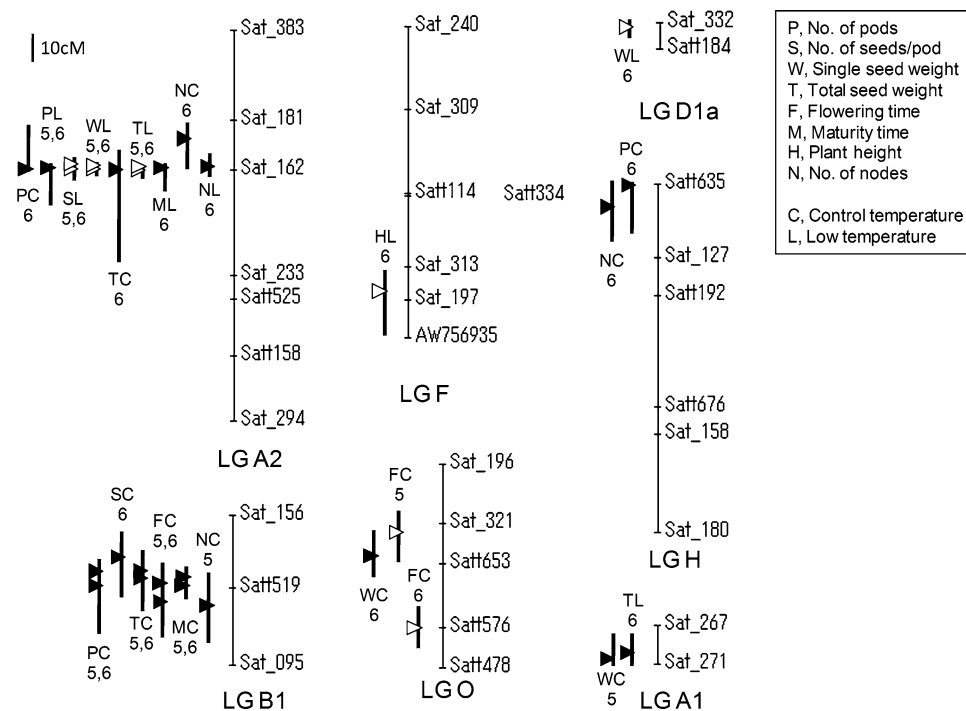[b] Markers designed in the present study are not included

Fig. 3 Linkage map and locations of quantitative trait loci (QTL) for traits associated with seed-yielding ability, maturing habit and plant architecture at control and low temperatures. Abbreviation of each trait and temperature regime is given in the *box*. Numbers 5 and 6 under the abbreviated trait name indicate that the QTL were identified in F₅ and F₆ generations, respectively. For example, WL56 indicates the QTL for single seed weight at low temperature, which was identified in the F₅ and F₆ generations. *Open triangles* indicate the positions of the LOD peaks of QTL with the Toyoharuka (TR) allele having a positive effect, while *closed triangles* represent the positions

of the LOD peaks of QTL with the Toyomusume (TM) allele having a positive effect. *Bars* indicate putative regions of QTL. A reduction of one LOD value from the LOD peaks was used to define borders of the confidence intervals. To avoid complexity, the regions of QTL for the same trait in F₅ and F₆ generations under the same temperature condition were integrated in one bar in the case of the confidence intervals being overlapped. Maturity time, plant height, and node number were not recorded for the F₅ RILs at low temperature. Linkage groups (LG) were designated according to Song et al. (2004). Linkage groups with no QTL are omitted from this figure

for each trait in the chilling environment in both generations (Fig. 3, Table 3). These QTL explained over 30 and 40% of total variance for seed number per pod and single seed weight, respectively. At these QTL the TR alleles had positive effects. QTL analysis for total seed weight per plant in the chilling environment also revealed a significant QTL near Sat_162 with the TR allele having positive effects, although a QTL of relatively small effect for pod number per plant was identified with the TR allele having negative effects. In addition, significant QTL for node number and maturity were identified near Sat_162 with the TM allele having positive effects. The observation of plants suggested that the effect of the TM allele at the QTL near Sat_162 on retarding maturity in the chilling environment was attributed to the slow discoloration of the pods containing no or small seeds. In the control environment, QTL of relatively small effect for pod number, total seed weight and node number were found near Sat_162 with the TM alleles having positive effects. As for other regions, significant QTL for several traits in the control environment were detected near Satt519 on LG B1. In this region, a QTL of a relatively large effect controlling maturity time was

identified, and this QTL probably influenced the other traits as well. However, no significant QTL were detected on LG B1 for the RILs grown in the chilling environment. Although several QTL were identified in other regions, the effects of the QTL were relatively small and of low reproducibility. These results indicated a central role for the genomic region near Sat_162 in chilling tolerance of TR. No QTL for flowering time or maturity time were detected on LGs C2, I or L, which confirmed that TR is of the same genotype at the *E1, E3 at E4* loci as TM (*e1E3E4*).

Since genes that have small effects could be missed to reduce false-positive QTL declarations (Holland 2007), comparison of mean values and ANOVA of the RILs with the Sat_162 marker class were performed to examine possible associations of the genomic region including Sat_162 with traits for which no significant QTL were identified by CIM. In the control environment, no or only a slight difference between the genotypes at Sat_162 was detected in flowering and maturity times, plant height, or yield components except for pod number (Table 4). The ovule numbers per pod were equal for both genotypes regardless of the temperature condition.

Table 3 List of quantitative trait loci (QTLs) identified for traits related to seed yield, maturity habit and plant architecture at control and low temperatures

Trait	Temp.	Generation	Threshold[a]	Position		LOD	r^2	Additive effect[d]
				LG[b]	Marker[c]			
No. of pods per plant	Control	F_5	2.8	B1	Satt519	6.1	0.20	−3.5
		F_6	2.6	A2	Sat_162	4.0	0.11	−2.9
				B1	Satt519	3.1	0.08	−2.5
				H	Satt635	2.8	0.06	−2.3
	Chilling	F_5	2.8	A2	Sat_162	4.2	0.10	−1.6
		F_6	2.7	A2	Sat_162	4.5	0.12	−2.0
No. of seeds per pod	Control	F_6	2.8	B1	Satt519	3.6	0.17	−0.09
	Chilling	F_5	2.8	A2	Sat_162	13.9	0.36	0.27
		F_6	2.8	A2	Sat_162	17.6	0.38	0.31
Single seed weight (mg)	Control	F_5	2.9	A1	Sat_271	3.2	0.09	−15
		F_6	2.8	O	Satt653	3.8	0.12	−21
	Chilling	F_5	2.8	A2	Sat_162	17.7	0.41	74
		F_6	2.7	A2	Sat_162	19.3	0.42	70
				D1a	Satt184	4.8	0.06	28
Total seed weight (g)	Control	F_5	2.7	B1	Satt519	4.0	0.10	−2.2
		F_6	2.7	A2	Sat_162	3.7	0.09	−2.2
				B1	Satt519	4.1	0.12	−2.6
	Chilling	F_5	2.8	A2	Sat_162	8.0	0.18	1.1
		F_6	2.8	A1	Sat_271	5.3	0.11	−0.9
				A2	Sat_162	15.5	0.31	1.6
Flowering time (day)	–	F_5	2.9	B1	Satt519	4.2	0.12	−0.44
				O	Sat_321	3.1	0.10	0.39
		F_6	2.8	B1	Satt519	3.6	0.11	−0.48
				O	Satt576	4.6	0.11	0.49
Maturity time[e] (day)	Control	F_5	2.8	B1	Satt519	6.2	0.18	−5.2
		F_6	2.8	B1	Satt519	9.6	0.22	−3.4
	Chilling	F_6	2.9	A2	Sat_162	6.2	0.15	−3.2
No. of nodes[e]	Control	F_5	2.8	B1	Satt519	4.9	0.18	−1.5
		F_6	2.9	A2	Sat_181	4.3	0.12	−1.4
				H	Satt635	3.8	0.14	−1.6
	Chilling	F_6	2.8	A2	Sat_162	4.6	0.12	−1.5
Plant height[e] (cm)	Control	F_6	2.8	B1	Satt519	3.0	0.09	−2.1
	Chilling	F_6	2.7	F	Sat_197	4.9	0.16	2.2

[a] Empirical LOD threshold value at the 0.05 alpha level by 1000 permutation test

[b] Linkage groups (LG) were designated according to Song et al. (2004)

[c] The marker closest to the QTL

[d] The additive effect indicates the effect of Toyoharuka (TR) alleles in comparison to a Toyomusume (TM) allele. Thus, when this value is negative, the TR allele reduces trait value

[e] Maturity time, plant height, and number of nodes were not recorded for the F_5 RILs at low temperature

Analysis with HIFs

To confirm the influence of the genomic region near Sat_162 on the traits associated with seed-yielding ability at low temperature and plant architecture, HIFs were developed. Although the distances among the markers near the QTL for chilling tolerance in this genomic region are quite large, the Sat_162 locus was computed to be very close to the QTL (Fig. 3). Therefore, the Sat_162 marker class was used for the development of HIFs. Since three-way ANOVA suggested the presence of genetic difference in the traits examined among families with little interaction with the genotype at Sat_162 (data not shown), a pairwise t test was used to estimate the differences in the effects of

Table 4 Mean values for the traits measured in the RILs of the Toyomusume (TM) and the Toyoharuka (TR) genotypes at SSR marker Sat_162

Temperature regime	Trait	F_5		F_6	
		Genotype at Sat_162		Genotype at Sat_162	
		TM	TR	TM	TR
Control	Days to flowering (day)	37	38	37	38[*a]
	Days to maturity (day)	117	114	124	124
	Plant height (cm)	93.4	92.4	78.4	78.5
	No. of branches	1.3	0.9	2.3	1.9[**]
	No. of pods on main stem	23.3	22.9	21.4	20.1
	No. of ovules per pod[b]	ND	ND	2.11	2.12
	No. of seeds per pod	1.77	1.84	1.60	1.66
	No. of seeds per pod on main stem	2.1	2.1	1.67	1.71
	Single seed weight (mg)	412	414	478	480
	Single seed weight for main stem (mg)	424	427	491	498
	Total seed weight for main stem (g)	18.6	18.2	17.8	16.8
Chilling	No. of ovules per pod[b]	ND	ND	1.97	1.97

[a] Significant at the levels of 0.05* and 0.01** by one-way ANOVA

[b] The trait was not measured in the F_5 generation

the genotypes at Sat_162. Total seed weight, seed number per pod and single seed weight were markedly different between the genotypes at Sat_162 in the chilling environment (Figs. 4, 1d). The TM genotype showed larger numbers of nodes and pods regardless of the temperature condition. These results were consistent with those for the RILs. Pod wall weight was larger for the TM genotype even in the chilling environment in contrast to seed weight. Similar results were obtained where the data for main stems were analyzed (Fig. 4).

Association between chilling tolerance and low-branching nature

To examine whether the chilling tolerance controlled by the QTL near Sat_162 was achieved as a result of low-branching nature and small pod number, correlation and regression analyses were performed for the F_6 RIL population grown in the chilling environment. Significant inverse correlations between the traits associated with chilling tolerance and the numbers of nodes and pods were found for the whole population (Table 5), although the r^2 values were less than those computed for the Sat_162 marker class, i.e., 0.57 for seed number per pod and 0.53 for single seed weight. Separation of the population according to the Sat_162 marker class reduced the levels of correlation, and no significant correlation was observed for the population composed of the TM genotypes. These results indicated that the genomic region near the Sat_162 locus accounted for a large part of the association between

the chilling tolerance and the natures of low-branching and setting fewer pods.

Discussion

QTL analysis for yield components revealed that the QTL with large effects for seed number per pod and single seed weight at low temperature are the major genetic factors associated with the chilling tolerance of TR. The seed number per pod should be influenced by ovule number per pod and fertilization efficiency. In the present study, seed abortion at early stages was also involved, since we did not count very small seeds. Considering that pod number per plant, which is dependent on fertilization efficiency, and ovule number per pod were not larger in the TR genotype at the QTL at low temperature, the genotypically differential values in seed number per pod at low temperature should be associated with genotypic difference in seed development efficiency. The QTL for seed number per pod and single seed weight at low temperature were mapped at almost the same position near Sat_162 between Sat_181 and Sat_233 on LG A2. Taken together, the results indicate that the QTL for seed number per pod and single seed weight in the chilling environment are probably the same locus, which controls seed development at low temperature.

In rice, several QTL associated with chilling tolerance at the reproductive phase have been identified (Saito et al. 2001; Takeuchi et al. 2001; Andaya and Mackill 2003b;
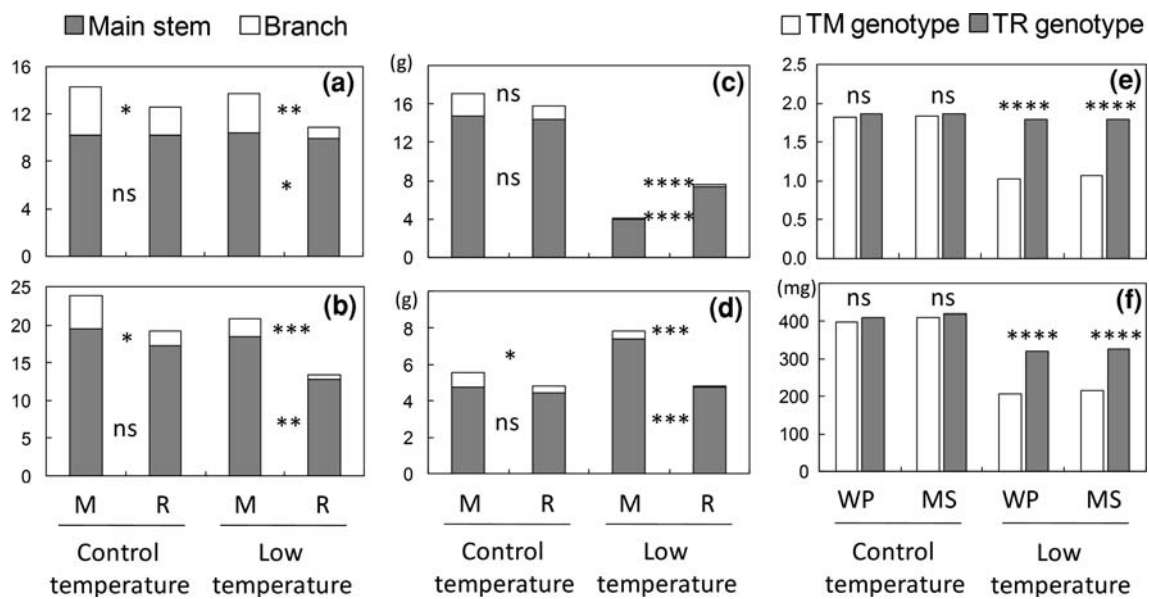
Fig. 4 Comparison of several traits between the lines of the Toyomusume (TM) and Toyoharuka (TR) genotypes at Sat_162 in heterogeneous inbred families at control and low temperatures. **a** Node number, **b** pod number, **c** total seed weight, **d** pod wall weight, **e** seed number per pod, **f** single seed weight. *Solid* and *open boxes* in **a–d** indicate the mean values for main stem and those for branches, respectively. *M* and *R* indicate the TM and the TR genotypes, respectively. *Open* and *solid boxes* in **e** and **f** indicate the mean values for the TM and TR genotypes, respectively. *Upper* and *lower asterisks* in **a–d** represent statistic significance for the whole plant and for the main stem, respectively. Significant at the levels of 0.05*, 0.01**, 0.001*** and 0.001**** by pairwise *t* test, respectively. *WP* and *MS* indicate whole plant and main stem

Table 5 Relationship between traits associated with chilling tolerance and plant architecture in the F_6 RIL population

Traits at low temperature		Whole population ($n = 156$)		TM genotype at Sat_162 ($n = 70$)		TR genotype at Sat_162 ($n = 70$)	
		No. of nodes	No. of pods	No. of nodes	No. of pods	No. of nodes	No. of pods
No. of seeds per pod	Corr. Coeff.[a]	−0.45****	−0.37****	−0.11 ns	−0.07 ns	−0.34**	−0.23 ns
	r^{2}[b]	0.20	0.13	0.00	−0.01	0.10	0.04
Single seed weight	Corr. Coeff.	−0.45****	−0.34****	−0.13 ns	0.05 ns	−0.21 ns	−0.16 ns
	r^2	0.20	0.11	0.00	−0.01	0.03	0.01

[a] Pearson's correlation coefficient. Significant at the levels of 0.01** and 0.0001****

[b] r^2 represents the value of adjusted r^2 that was calculated using the REG procedure (SAS Institute 1996)

Kuroki et al. 2007). All of them have been reported to condition fertility of spikelets at low temperature, which is mainly determined by tolerance of the male reproductive organ (Satake and Hayase 1970). In soybean, the major QTL near the *T* locus associated with chilling tolerance at the reproductive phase was shown to influence every yield component as well as the vegetative growth at low temperature (Kurosaki et al. 2004; Funatsuki et al. 2005). Other minor QTL were likely to be associated with fertilization (Funatsuki et al. 2005). In contrast, the QTL near Sat_162 found in the present study appeared to play a crucial role specifically in seed development at low temperature. The larger value in pod wall weight in the TM genotype at Sat_162 relative to that in the TR genotype in the HIFs suggests that even general sink strength at low temperature is unaffected by this QTL. Thus, a new type of

chilling tolerance gene may underlie the QTL. Pyramiding the tolerance allele at the QTL near Sat_162 and those at the QTL reported previously may be effective to attain a higher level of chilling tolerance.

Some genes conferring tolerance to environmental stress have been demonstrated to influence plant growth even under non-stress conditions (e.g., Shan et al. 2007). Analyses with the RILs and HIFs in the present study revealed the possible influence of the genomic region including Sat_162 on other traits. The nature of fewer nodes on branches and relatively small pod number per plant in TR was also observed under relatively low planting-density conditions in the field, while TR exhibits a pod number per plant comparable to that of TM under high planting-density conditions (Shirai et al. 2004). Analyses on the traits of main stem in the present study indicate that the chilling

tolerance conferred by the TR allele at the QTL is not due to the plant architecture of fewer branch nodes that develop in a large part after flowering, i.e., the time of starting chilling treatment. Correlation and regression analyses suggest that pod number is not the primary factor determining the seed number per pod and single seed weight at low temperature. Therefore, the QTL may be useful under field conditions in which TR displays a pod number similar to that of TM. In addition, no association between the QTL and maturing habit is also an advantage of this QTL, unlike other QTL found in soybean (Funatsuki et al. 2005). However, it is possible that the same locus influences chilling tolerance and plant architecture since some genes such as ones involved in hormonal regulations may affect both traits simultaneously (Penfield 2008). It would be interesting to clarify in a future study whether the traits of chilling tolerance and low-branching are controlled pleiotropically by the same genetic factor or by different tightly linked genes. In addition, it is important to confirm the effects of these QTLs in the field in the future.

Map-based cloning of QTL is a useful tool to unravel molecular mechanisms underlying tolerance to environmental stress (Ren et al. 2005; Xu et al. 2006). The QTL identified in the present study could be a target for fine-mapping and map-based cloning because of the magnitude of its effect. However, we could not find polymorphic SSR markers in the genomic region between Sat_181 and Sat_233 except for Sat_162, although we tested 14 SSR markers that were designed on the basis of the public genomic sequence (DoE Joint Genome Institute, http://www.jgi.doe.gov/News/news_1_17_08.html) in addition to the 14 markers published by Song et al. (2004) (data not shown). Considering that TM and TR have genealogically common ancestors, the genomic region between Sat_181 and Sat_233 except for a small range including the Sat_162 locus and the QTL may be identical in TM and TR. It will be necessary to create a mapping population derived from a cross between TR and another accession displaying high polymorphism in the genomic sequence of the region of interest. Identification of the gene responsible will provide new insights into the mechanism underlying the chilling tolerance as well as into seed development.

Since tolerance to environmental stress is typically controlled by multiple genetic loci (Leung 2008), QTL analysis has been employed in many studies to identify genetic factors associated with tolerance to environmental stress. While most QTL had relatively small effects, a few QTL have been demonstrated to have sufficiently large effects for MAS (Xu et al. 2006). In the present study, we also could identify a QTL with a large effect on chilling tolerance at the seed-yielding level. Various environmental stresses reduce seed number per pod and seed size in

legume plants (Desclaux et al. 2000; Awal and Ikeda 2003; Karim et al. 2003; Rainey and Griffiths 2005; Munoz-Perea et al. 2006). The findings in the present study may encourage legume breeders to conduct genetic screening and QTL analysis for tolerance to environmental stresses.

References

Andaya VC, Mackill DJ (2003a) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. J Exp Bot 54:2579–2585

Andaya VC, Mackill DJ (2003b) QTL conferring cold tolerance at the booting stage of rice using recombinant inbred lines from a japonica x indica cross. Theor Appl Genet 106:1084–1090

Awal MA, Ikeda T (2003) Controlling canopy formation, flowering, and yield in field-grown stands of peanut (Arachis hypogaea L.) with ambient and regulated soil temperature. Field Crop Res 81:121–132

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trend Plant Sci 12:444–451

Churchill G, Doerge R (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Desclaux D, Huynh TT, Roumet P (2000) Identification of soybean plant characteristics that indicate the timing of drought stress. Crop Sci 40:716–722

Fehr WE, Caviness CE, Burmood PT, Pennington J (1971) Stage of development description of soybeans, Glycine max (L.) Merrill. Crop Sci 11:929–931

Funatsuki H, Matsuba S, Kawaguchi K, Murakami T, Sato Y (2004) Methods for evaluation of soybean chilling tolerance at the reproductive stage under artificial climatic conditions. Plant Breed 123:558–563

Funatsuki H, Kawaguchi K, Matsuba S, Sato Y, Ishimoto M (2005) Mapping of QTL associated with chilling tolerance during reproductive growth in soybean. Theor Appl Genet 111:851–861

Funatsuki H, Hajika M, Hagihara S, Yamada T, Tanaka Y, Tsuji H, Ishimoto M, Fujino K (2008) Confirmation of the location and the effects of a major QTL controlling pod dehiscence, qPDH1, in soybean. Breed Sci 58:63–69

Holland JB (2007) Genetic architecture of complex traits in plants. Curr Opin Plant Biol 10:156–161

SAS Institute (1996) SAS/STAT user's guide, vols 1 and 2, version 6, 4th edn. Cary, USA

Jompuk C, Fracheboud Y, Stamp P, Leipner J (2005) Mapping of quantitative trait loci associated with chilling tolerance in maize (Zea mays L.) seedlings grown under field conditions. J Exp Bot 56:1153–1163

Karim A, Fukamachi H, Komori S, Ogawa K, Hidaka T (2003) Growth, yield and photosynthetic activity of Vigna radiata L. Grown at different temperature and light levels. Plant Prod Sci 6:43–49

Kuroki M, Saito K, Matsuba S, Yokogami N, Shimizu H, Ando I, Sato Y (2007) A quantitative trait locus for cold tolerance at the

booting stage on rice chromosome 8. Theor Appl Genet 111:593–600

Kurosaki H, Yumoto S (2003) Effects of low temperature and shading during flowering on the yield components in soybeans. Plant Prod Sci 6:17–23

Kurosaki H, Yumoto S, Matsukawa I (2004) Correlation of cold-weather tolerance with pubescence color and flowering time in yellow hilum soybeans in Hokkaido. Breed Sci 54:303–311

Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Leipner J, Jompuk C, Camp KH, Stamp P, Fracheboud Y (2008) QTL studies reveal little relevance of chilling-related seedling traits for yield in maize. Theor Appl Genet 116:555–562

Leung H (2008) Stressed genomics—bringing relief to rice fields. Curr Opin Plant Biol 11:201–208

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158

Manly KF, Cudmore RH Jr, Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 12:930–932

Morrison MJ, Voldeng HD, Guillemette RJD (1994) Soybean pubescence color influences seed yield in cool-season climates. Agron J 86:796–799

Munoz-Perea CG, Teran H, Allen RG, Wright JL, Westermann DT, Singh SP (2006) Selection for drought resistance in dry bean landraces and cultivars. Crop Sci 46:2111–2120

Palmer RG, Pfeiffer TW, Buss GR, Kilen TC (2004) Qualitative genetics. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses, 3rd edn. American Soc Agron Inc Crop Sci Soc America Inc Soil Sci Soc America Inc Publishers, Madison, pp 137–233

Penfield S (2008) Temperature perception and signal transduction in plants. New Phytol 179:615–628

Rainey KM, Griffiths PD (2005) Diallel analysis of yield components of snap beans exposed to two temperature stress environments. Euphytica 142:43–53

Raper CD Jr, Kramer PJ (1987) Stress physiology. In: Wilcox JR (ed) Soybeans: improvement, production, and uses, 2nd edn. American Soc Agronomy, Inc. Crop Sci Soc America. Inc. Soil Sci Soc America Inc. Publishers, Madison, pp 589–642

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37:1141–1146

Saito K, Miura K, Nagano K, Hayano-Saito Y, Araki H, Kato A (2001) Identification of two closely linked quantitative trait loci for cold tolerance on chromosome 4 of rice and their association with anther length. Theor Appl Genet 103:862–868

Satake T, Hayase H (1970) Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimation of pollen developmental stage and the most sensitive stage to coolness. Proc Crop Sci Soc Jpn 39:468–473

Shan DP, Huang JG, Yang YT, Guo YH, Wu CA, Yang GD, Gao Z, Zheng CC (2007) Cotton GhDREB1 increases plant tolerance to low temperature and is negatively regulated by gibberellic acid. New Phytol 176:70–81

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611

Shirai S, Yumoto S, Matsukawa I, Tanaka Y, Hagihara S, Kurosaki H, Sumida S, Yamazaki T, Suzuki C, Ohnishi S (2004) A new soybean variety "Toyoharuka" (in Japanese). http://www.affrc.go.jp/ja/research/seika/data_nics/h16/d16006

Song QJ, Marek LF, Shoemaker RC, Lark KG, Concibido VC, Delannay X, Specht JE, Cregan PB (2004) A new integrated genetic linkage map of the soybean. Theor Appl Genet 109:122–128

Takahashi R, Asanuma S (1996) Association of t gene with chilling tolerance in soybean. Crop Sci 36:559–562

Takahashi R, Benitez ER, Funatsuki H, Ohnishi S (2005) Soybean maturity and pubescence color genes improve chilling tolerance at high latitude regions. Crop Sci 45:1387–1393

Takeuchi Y, Hayasaka H, Chiba B, Tanaka I, Shimano T, Yamagishi M, Nagano K, Sasaki T, Yano M (2001) Mapping quantitative trait loci controlling cool-temperature tolerance at booting stage in temperate Japonica rice. Breed Sci 51:191–197

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Ann Rev Plant Physiol Plant Mol Biol 50:571–599

Tuinstra MR, Ejeta G, Goldsbrough PB (1997) Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. Theor Appl Genet 95:1005–1011

Wang SCJ, Basten CJ, Zeng Z-B (2005) Windows QTL cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC, USA

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ (2006) Sub1a is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442:705–708

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Ann Rev Plant Biol 57:781–803

Yamanaka N, Ninomiya S, Hoshi M, Tsubokura Y, Yano M, Nagamura Y, Sasaki T, Harada K (2001) An informative linkage map of soybean reveals QTL for flowering time, leaflet morphology and regions of segregation distortion. DNA Res 8:61–72

Yamanaka N, Watanabe S, Toda K, Hayashi M, Fuchigami H, Takahashi R, Harada K (2005) Fine mapping of the FT1 locus for soybean flowering time using a residual heterozygous line derived from a recombinant inbred line. Theor Appl Genet 110:634–639

Yin X, Struik PC, Kropff MJ (2004) Role of crop physiology in predicting gene-to-phenotype relationships. Trend Plant Sci 9:426–432