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RESEARCH

Selection for Cold Tolerance during Flowering in Short-Season Soybean

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ABSTRACT

Cold temperatures during early reproductive development may result in reduced pod and seed formation in soybean [*Glycine max* (L.) Merr.]. The objectives of this work were to quantify cold tolerance of six potential parents, to screen breeding populations for cold tolerance, to search for associations between molecular markers and cold tolerance phenotypes, and to evaluate field performance of breeding lines. Six cultivars were evaluated for pod and seed set after exposure to 0 to 6 wk cold periods (15/5°C day/night), which began at flowering in growth cabinets. Evaluation at 6 vs. 2 wk following the end of a 3 or 6 wk cold period best discriminated between cold tolerant (CT) and cold sensitive (CS) cultivars. Two recombinant inbred line populations were cold stressed in growth cabinets with divergent selection performed over the F_5 through F_7 generations to select CT and CS lines. Bulked segregant analysis identified six chromosomal regions, four of which are related by homology, as potentially impacting the trait. Over 8 yr in the field at Ottawa, Canada, CT lines yielded 379 kg ha^{-1} more but matured 5.5 d later than CS lines without visual symptoms of cold damage. Following selection for early maturity, random lines from an additional four populations developed from a selected CT and CS line each crossed to another CT and CS parent were field tested in three environments. Cold tolerant × CT lines yielded about 8% more than CS × CS lines, again without visual cold damage symptoms.

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Abbreviations: BSA, bulked segregant analysis; CS, cold sensitive; CT, cold tolerant; LG, linkage group; MAD, modified augmented design; NIL, near isogenic line; NPBP, near-perfect bulked segregant analysis banding pattern; PBP, perfect bulked segregant analysis banding pattern; PP, parthenocarpic pod; PVP, U.S. plant variety protection certificate number; QTL, quantitative trait locus; RIL, recombinant inbred line; SSR, simple sequence repeat.

Soybean can suffer from cold temperatures occurring during reproduction. In Hokkaido, Japan, mean seed yields were associated with mean summer temperatures (Funatsuki and Ohnishi, 2009). Low temperatures before and throughout the flowering period can result in reduced pod and seed formation (Gass et al., 1996; Kurosaki et al., 2003; Lawn and Hume, 1985; Saito et al., 1970) or malformed parthenocarpic pods (Hume and Jackson, 1981). Past work evaluating cold sensitivity usually involved imposition of cool or cold temperatures before (Ohnishi et al., 2010; Saito et al., 1970) or at the onset of flowering (Funatsuki et al., 2004; Hume and Jackson, 1981; Kurosaki et al., 2003; Lawn and Hume, 1985). The response to cold stress was quantified by counting pods or seeds (Gass et al., 1996; Hume and Jackson, 1981; Kurosaki et al., 2003; Lawn and Hume, 1985; Saito et al., 1970), measuring pod elongation (Ohnishi et al., 2010), noting parthenocarpic pod formation (Hume and Jackson, 1981), or measuring

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seed yield (Funatsuki et al., 2004). While some researchers provided a cold period as short as 1 wk (Ohnishi et al., 2010), a review suggested that low temperatures for 4 wk or longer are most useful to discriminate among genotypes (Funatsuki and Ohnishi, 2009). Cold night temperatures in controlled environments used for evaluation varied among research groups with the lowest reported values of 8 (Lawn and Hume, 1985) or 7°C (Hume and Jackson, 1981; Schmid and Keller, 1980). Field trials have also been used, with naturally occurring cold temperatures, to evaluate cold stress (Funatsuki et al., 2004; Gass et al., 1996).

Genetic and physiological studies using tolerant and susceptible varieties and near isogenic lines (NILs) have associated chilling tolerance with the well-characterized genetic loci *cAPX1*, *T*, *Ln*, *P1*, and *Dt1* as well as with the three maturity loci *E1*, *E3*, and *E4* (reviewed by Funatsuki and Ohnishi, 2009; Toda et al., 2011). The *T* locus was reported to be related to seed yield in cool seasons when comparing tawny and gray pubescent lines (Morrison et al., 1994, 1997). Quantitative trait locus (QTL) analysis in a segregating recombinant inbred population identified the *E1* and linked *T* genomic region, the *E3* locus, and a genomic region near simple sequence repeat (SSR) marker Satt635 on linkage group (LG) H as important in that population (Funatsuki et al., 2005). In a related population, a new QTL was identified near SSR marker Sat_162 on LG A2 (Ikeda et al., 2009). Whether additional loci influencing this trait exist or not, the above nine loci demonstrate that the genetics of chilling tolerance in soybean is multigenic and complex. Knowledge of the loci involved and their allelic status in breeding lines would facilitate the use of molecular markers to assist in the development of chilling tolerant varieties.

The objectives of this work were to quantify cold tolerance of potential parents, to screen breeding populations for cold tolerance, to search for associations between molecular markers and cold tolerance phenotypes, and to evaluate the field performance of breeding lines.

MATERIALS AND METHODS

Growth Cabinet Evaluation of Cold Tolerance

Six short-season soybean cultivars were used to evaluate cold tolerance screening methods. Maple Arrow was used since it was previously found to be tolerant to lower temperatures at podding (Hume and Jackson, 1981). Other cultivars studied included 9063 (Voldeng et al., 1997) developed by Agriculture and Agri-Food Canada at Ottawa, Evans (Lambert and Kennedy, 1975) developed in Minnesota, and 9007 (U.S. plant variety protection certificate number [PVP] 9300244), 9008 (PVP 9500028), and 9071 (PVP 9300243) developed by Pioneer Hi-Bred. Seeds were germinated in vermiculite. After cotyledon emergence, individual seedlings were transplanted into 12.7 cm pots containing a sterilized soil mix in a ratio of 3:2:1:2:2 of loam, peat moss, sand, vermiculite, and crushed brick, respectively. Plants were grown in growth cabinets under 14 h days with a 25/20°C day/night temperature regime. When all the plants in a growth cabinet were flowering, the highest flowering node on each plant was tagged and a 15/5°C day/night temperature regime was imposed for 0, 1, 3 or 6 wk. After the cold treatment ended, plants were moved to a greenhouse with a 14-h day provided by high pressure sodium lamps and 25/20°C day/night temperature regime. At both 2 and 6 wk after the end of the cold treatment, a number of observations were made. The numbers of developing seeds on the next four nodes above the tagged node were counted. A parthenocarpic pod (PP) score was observed in which 1 indicates no PP, 2 indicates long lasting flowers or dead and adhering flowers or buds, 3 indicates few PP, 4 indicates many PP, and 5 indicates PP over the full length of the stem and very few pods with seeds. A pod set score was observed in which 1 indicates no barren nodes, 2 indicates one barren node and warm nodes well podded, 3 indicates two to three barren nodes and warm nodes well podded, 4 indicates some barren nodes and generally reduced podding, and 5 indicates many barren nodes and only a few one-to-two-seeded pods. Warm nodes were those that flowered after the end of the cold treatment. A podding index developed for this study was calculated as podding index = PP score × pod set score.

Cold Screening of Recombinant Inbred Line Populations

Two populations were developed for cold screening: X3805 (9063 × 9007) and X3815 (OT92-14 × 9007). OT92-14 has the pedigree PI 196529/6*Maple Arrow/3/Mandarin/PI 438477//OX611. Populations were advanced to the F_4 generation using single seed descent. Random F_5 seeds and parents were planted and screened for cold tolerance (138 plants for X3805 and 151 plants for X3815). Plants were grown as described above and then cold stressed for 2 wk and scored 6 wk following the end of the cold treatment for seed number and PP score. Selected cold tolerant (CT) and cold sensitive (CS) plants were harvested and retested. The second screening used two replicates (pot with single plant as experimental unit) for each line (36 lines for each population) with each population in a separate test. Plants were cold stressed for 3 wk and scored 6 wk following the end of the cold treatment for seed number, PP score, pod set score, and podding index. Selected CT and CS plants were harvested and retested. The third screening used two replicates for each line (16 lines for X3805 and 10 lines for X3815) with both populations in the same test. Plants were cold stressed for 3 wk and scored 6 wk following the end of the cold treatment for seed number, PP score, pod score, and podding index. Experimentwise standard errors were calculated and results were reported graphically.

Molecular Marker Analysis

The most tolerant and the most susceptible lines in both the X3805 and X3815 populations were studied using the bulked segregant analysis (BSA) strategy of Michelmore et al. (1991). Bulked segregant analysis is based on the hypothesis that a bulk of the most tolerant lines should be homogeneous for the tolerant allele at the locus or few loci for tolerance and should be heterogeneous at all unlinked and unselected loci and reciprocally for a susceptible bulk. Therefore, genotyping the bulks of selected lines should identify loci tightly linked to the gene or

genes conditioning simply inherited traits. Samples of DNA were isolated from the 16 lines from X3805 that were used for the third screening for chilling tolerance, and the DNA from the eight tolerant lines was mixed to create a tolerant bulk DNA sample and from the eight sensitive lines to create a sensitive bulk DNA sample. The probability that all eight lines in a bulk carry the same allele at an unselected locus by random chance is $(0.5)^7 = 7.81 \times 10^{-3}$ and that the two bulks do so for contrasting alleles that are also in the correct tolerance or susceptibility phase with the parents is $0.5 \times 0.5 \times (0.5)^7 \times (0.5)^7 = 1.53 \times 10^{-5}$. Similarly, a tolerant bulk DNA sample based on five lines and a sensitive bulk DNA sample based on four lines (since one DNA sample was lost) were created from the 10 lines from the X3815 population that were used for the third screening. The probability of the X3815 bulks giving a perfect correlation in BSA by random chance is 1.95×10^{-3}. Therefore, the probability of false positives is low. Samples of DNA were isolated from leaves using a $CO(NH_2)_2$ (urea) extraction buffer method (Molnar et al., 2003). The four bulk DNA samples as well as DNA samples from the two CT parents (9063 and OT92-14) and common susceptible parent (9007) were genotyped with approximately 390 SSR markers selected from the soybean consensus map (Song et al., 2004) to achieve broad genome coverage. The polymerase chain reaction method and silver stained acrylamide gel electrophoresis procedures were followed as described in Molnar et al. (2003). Banding patterns were compared manually and SSRs noted where the tolerant and susceptible parents were polymorphic (described here as A vs. B banding patterns) and the tolerant and susceptible bulks "perfectly" reflected the same polymorphism (A vs. B). Simple sequence repeats were also noted where the banding pattern in the bulks (for example strong A band vs. strong B plus weak A bands) was not random (would expect both A and B bands in both bulks, i.e., AB vs. AB) and "near-perfectly" reflected that of the parents. Such near-perfect patterns can occur for a variety of reasons, the most obvious being when one of the lines in a bulk carries the opposite allele, as is expected for SSRs more loosely linked to the target locus, or if any of the recombinant inbred line (RIL) genotypes are heterozygous. For efficiency, in this manuscript perfect bulked segregant analysis banding pattern (PBP) and near-perfect bulked segregant analysis banding pattern (NPBP) will be used to describe the two types of informative SSR banding patterns or the SSR marker producing that pattern and are not intended to reflect any other attributes. The position in the soybean genome of each SSR identified by BSA was identified using the genomic sequence (assembly version 1.01) available on SoyBase at http://www.soybase.org/ (accessed 23 Apr. 2012). Regions syntenic to each of these SSR markers were identified using the duplicated regions feature of the same database.

Field Testing of Cold Screened Lines

Selected lines from populations X3805 (seven CT and eight CS) and X3815 (five CT and five CS) and their parents were yield tested in Ottawa for 8 yr (1998 to 2006, excluding 2005). The experiments were planted from 15 to 30 May (25 May 1998, 17 May 1999, 20 May 2000, 15 May 2001, 22 May 2002, 21 May 2003, 17 May 2004, and 30 May 2006) at a rate of 50 seeds m^{-2}. Soil samples were taken before seeding and P and K were broadcast and incorporated before planting based on soil test recommendations from provincial agriculture extension publications. Plot sizes were 1.6 by 5 m consisting of four rows spaced 0.4 m apart. All plots were prepared with fall tillage with a chisel plow and spring tillage with a cultivator and harrow followed by a combination cultivator–packer. Weeds were controlled using herbicides. Days to maturity and plant height were recorded for each plot. All four rows in a plot were combine harvested, the seed air dried, and seed yield reported at 130 g kg^{-1} moisture. Mass was determined for 100 seeds from each plot and used as a measure of seed size. Seed protein and oil were determined by near-infrared transmission spectroscopy (Infratec 1241 Grain Analyzer; Foss North America, Inc.). Maximum and minimum daily temperatures were observed at the experiment station weather station. Daily growing degree days were calculated as [(Tmax + Tmin)/2 − Tbase], with Tmax equal to daily maximum temperature, Tmin equal to daily minimum temperature, and Tbase equal to a base temperature of 10°C. Daily growing degree days were summed for the month of July to characterize the flowering period.

Experiments were arranged in a lattice design with two replications. Analysis of variance was performed over years using only complete blocks with PROC MIXED of SAS (SAS Institute, 2002). In these analyses, year, replication within year, and interactions including year were considered random effects while other effects were considered fixed. Significance was determined at the 5% level unless otherwise indicated. Least square means were calculated with PROC GLM of SAS. Standard errors and coefficients of variation were reported as measures of variability.

Field Testing of Random Lines from Structured Populations

One CT (86-CT) and one CS (138-CS) line of similar maturity were selected from the X3805 population and each was crossed to a CT (OT99-8) and CS (9007) parent for a total of four populations. OT99-8 is an unreleased, early maturing line with the pedigree Maple Belle/AC Bravor (Voldeng et al., 1996a)// AC Harmony (Voldeng et al., 1996b). These populations were advanced using single seed descent and F_5 rows were bulked in 2008 for further testing. The populations were evaluated in modified augmented design (MAD) trials (Lin and Poushinsky, 1985) at the Central Experimental Farm, Ottawa, ON, in 2009. Lines maturing equivalent to or earlier than our medium maturity check 'Maple Glen' (Voldeng et al., 1996c) and therefore considered adapted to Manitoba were retained and tested further in two replicate randomized complete block design tests at the Central Experimental Farm, Ottawa, ON, and the Agriculture and Agri-Food Canada research station in Morden, MB, in 2010. Trials were planted on 25 May 2009 and on 21 May 2010 in both Ottawa and Morden. Field trial procedures were as reported above for the first set of field trials. Mean seed yield and other agronomic characters were calculated from MAD adjusted means from 2009 and from the two locations grown in 2010.

RESULTS AND DISCUSSION

Cold Screening

Subjecting soybean cultivars to cold temperatures at the onset of flowering generally reduced pod and seed set and the reduction was greater as the cold treatment lengthened

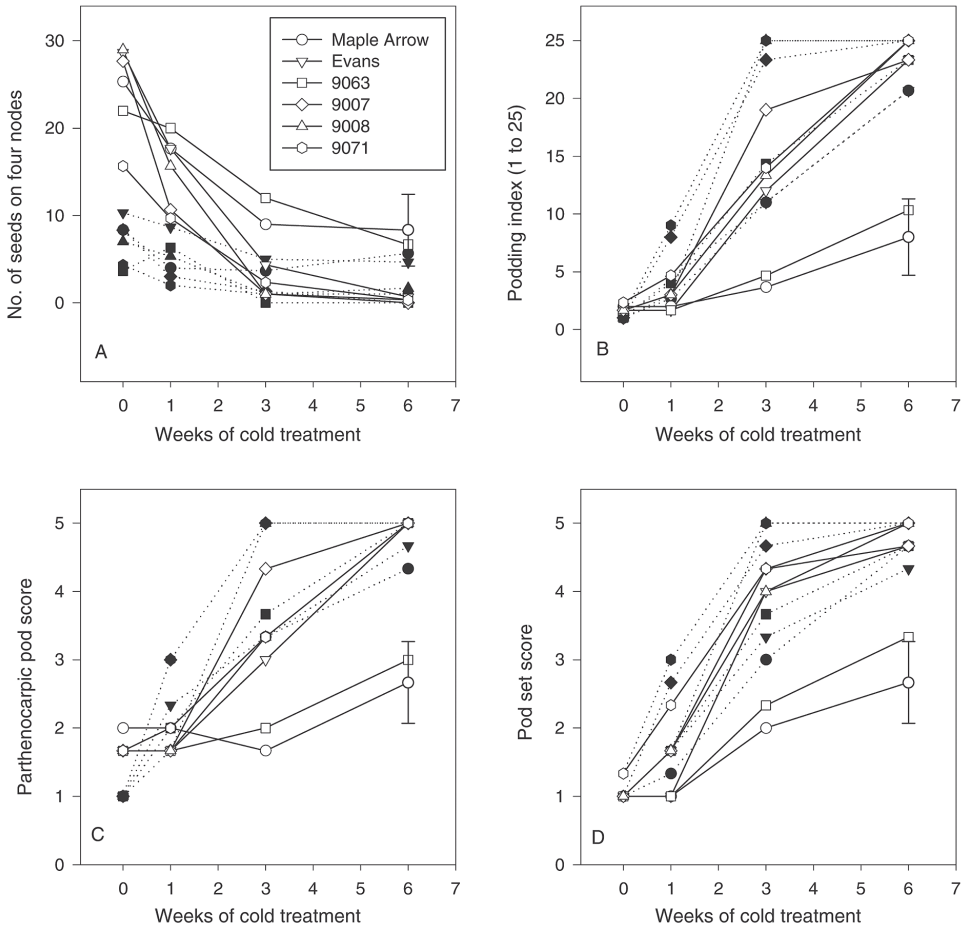

Figure 1. Mean seed numbers (A), podding index (B), parthenocarpic pod (C), and pod set (D) scores for soybean cultivars subjected to cold treatments, in a growth cabinet, for various lengths of time following the start of flowering. Filled symbols were scored at 2 wk following the end of the cold treatment while open symbols were scored 6 wk following the end of the cold treatment. The experimentwise error bar shows 2 SE.

(Fig. 1). Two cultivars were observed to have greater tolerance to cold treatments, Maple Arrow and 9063; however, this tolerance was seen only when the lines were allowed sufficient time to recover from the cold; that is, observations 2 wk following the end of the cold treatment would result in these cultivars being classified as CS similar to the other cultivars while observations 6 wk following the end of the cold treatment differentiated Maple Arrow and 9063 from the other four cultivars. There are no direct comparisons of scoring over time in the literature; however, observation of plant seed yield (Kurosaki et al., 2003; Saito et al., 1970) is a delayed observation. Cold treatments lasting 3 and especially 6 wk following the onset of flowering were most useful to discriminate cold tolerance similar to comparisons of 2 and 4 wk of cold treatment (Kurosaki et al., 2003) or 4 wk or longer (Funatsuki and Ohnishi, 2009).

Two F_5 RIL populations (X3805 [9063 × 9007] and X3815 [OT92-14 × 9007]) were screened for cold tolerance for 2 wk at the onset of flowering and scored 6 wk following the end of the cold treatment for PP score. These two populations exhibited a continuous distribution for the PP score (Fig. 2) similar to the continuous distribution of

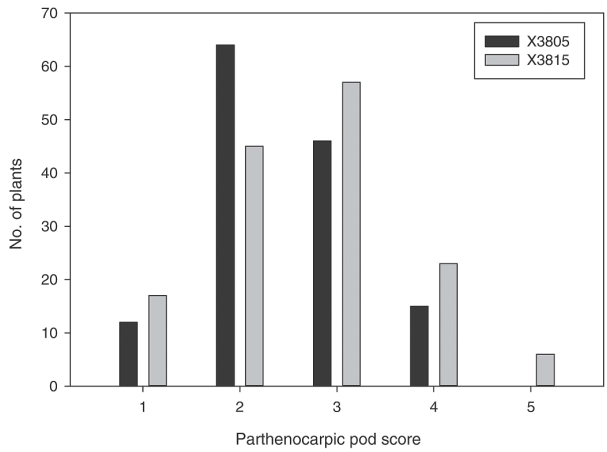

Figure 2. Histogram of parthenocarpic pod score for individual F_5 soybean plants from two crosses (X3805 [9063 × 9007] and X3815 [OT92-14 × 9007]) scored 6 wk after the end of a 2 wk cold treatment in a growth cabinet, which began at first flower.

the chilling tolerance index in a RIL population (Funatsuki et al., 2005). Parthenocarpic pods were previously considered a useful indicator of cold damage in many genotypes (Hume and Jackson, 1981). In the first screening, 2 wk of

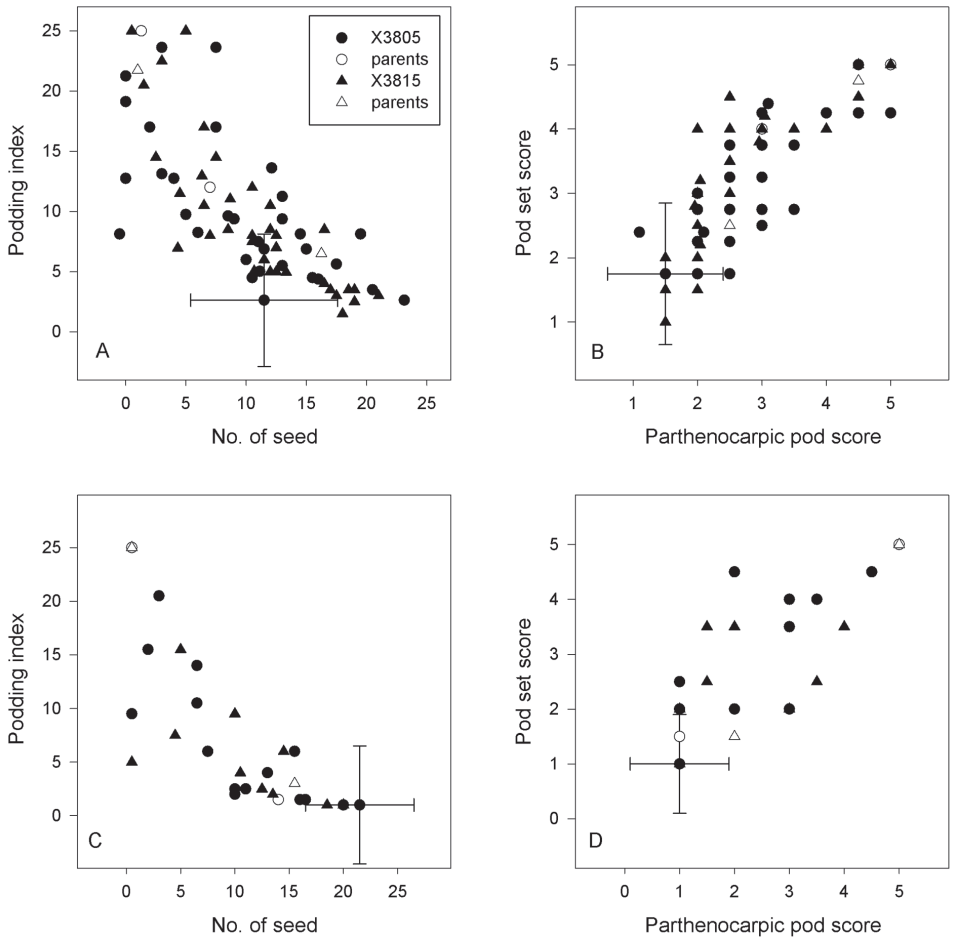

Figure 3. Mean podding index, seed number, pod set score, and parthenocarpic pod score scored 6 wk after the end of a 3-wk growth cabinet cold treatment, which began at first flower, in the F_6 (A and B) and F_7 generation (C and D) of selected soybean lines from two crosses (X3805 [9063 × 9007] and X3815 [OT92-14 × 9007]). Error bars show 2 SE.

cold treatment were used to manage larger numbers of lines while in the following two generations of testing, the cold treatment was applied for 3 wk to increase the ability to discriminate the cold response. After each generation of cold treatment, equivalent numbers of lines were selected from each of the CS and CT tail of the population and retested. For each trait, pod set, PP score, podding index, and number of seed set, the resulting lines showed a continuous distribution in both the F_6 and F_7 generation (Fig. 3). In a comparison of seed number and podding index over the F_6 and F_7 generations, it was seen that some observations, especially of CT lines, were variable (Fig. 4). Multiple cold screening tests were required to reliably identify CT lines.

Bulk Segregant Analysis

For both the X3805 and X3815 populations, BSA (Michelmore et al., 1991) was used to compare the genotypes of DNA bulks constructed from the most tolerant and the most susceptible lines to identify SSR markers that may be linked to the gene or genes responsible for this trait. A prerequisite for BSA is that the parental lines are polymorphic for a particular marker; for example, the tolerant parent producing an A banding pattern and the susceptible parent producing a B banding pattern, each diagnostic of an alternate allele. If the parental alleles are randomly distributed among the lines constituting each bulk, then each bulk would be expected to project a double banding pattern (AB). If all lines in the tolerant bulk have the tolerant parent's allele, the tolerant bulk would be expected to display a pure A banding pattern and likewise the susceptible bulk a pure B banding pattern. For convenience, instances of the latter will be referred to as perfect bulked segregant analysis banding pattern (PBP) and used to describe correlations or banding patterns or markers in this manuscript. Instances where the banding patterns observed in the bulks did not exactly reflect the parents but also were not random (for example the tolerant bulk having a strong A band and the susceptible bulk having both a strong B band and a weak A band) will be referred to as near-perfect bulked segregant analysis banding pattern (NPBP). In the context of the BSA study, neither the term PBP nor NPBP is intended to reflect any other attributes of such markers or banding patterns. In X3805, a total of 390 SSR markers were selected to give broad genome coverage and of these 223 were polymorphic on the parents. For three and 37 SSR markers, respectively, the polymorphism was PBP or NPBP in the

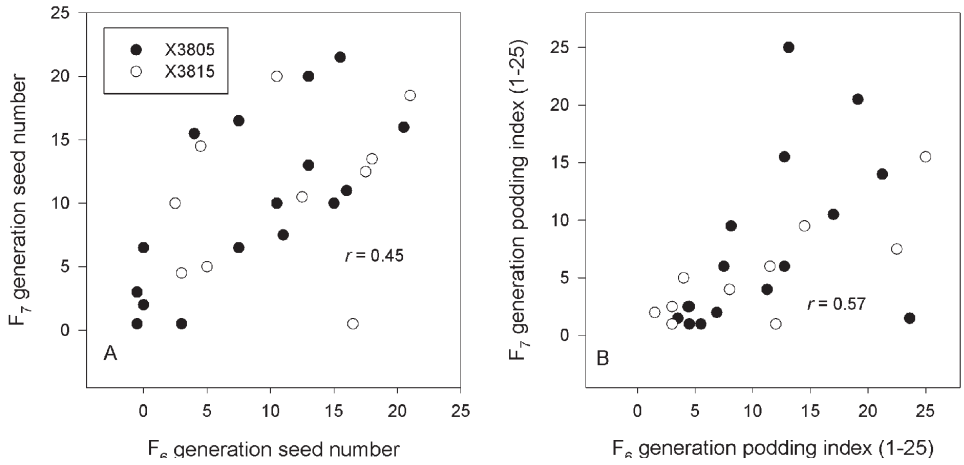

Figure 4. Correlation of seed number (A) and podding index (B) scored 6 wk after the end of a 3-wk growth cabinet cold treatment, which began at first flower, in the F_6 and F_7 generations of soybean lines from two crosses (X3805 [9063 × 9007] and X3815 [OT92-14 × 9007]) selected for cold sensitivity or cold tolerance.

bulks. In X3815, a total of 391 SSR markers were selected of which 207 were polymorphic on the parents. For 2 and 32 SSR markers, respectively, the polymorphism was PBP or NPBP in the bulks.

The five SSRs that gave a PBP correlation across the parents and bulks of one or the other population identified five loci on four LGs (Table 1). An additional six SSRs exhibited a NPBP correlation simultaneously in both of these independently developed populations. They plus another SSR (Satt253), which gave a PBP correlation in X3815 and NPBP in X3805, may be associated with susceptibility genes contributed by the common susceptible parent (9007). Together, these PBP and NPBP markers map to six chromosomes on the genome sequence-based physical map (Table 1).

Satt216 on chromosome 2 (LG D1b) gave a PBP correlation in X3805 and clusters with NPBP marker Satt095 in X3815 (Table 1). Satt095 (at position 6.307 Mb on the genome sequence physical map) is tightly linked to a sequence (at position 7.282 Mb) orthologous to marker Sat_271, which is the peak marker for a QTL (WC5) on chromosome 5 (LG A1) for single seed weight in control lines and a QTL (TL6) for total seed weight following reproductive chilling (Ikeda et al., 2009). Satt095 is also linked to a sequence orthologous to Sat_321, the peak marker for a QTL (FC5) for flowering time under control conditions in the reproductive chilling study of Ikeda et al. (2009).

A large portion of chromosome 6 (LG C2) may be important in chilling tolerance. Satt291 gave a PBP correlation in X3805 and clusters with multiple NPBP markers in both populations, of which Satt227 and Satt520 are NPBP in both populations (Table 1). Further down the chromosome, Satt489 is PBP in X3805 and clustered with markers Satt100, Satt319, and Satt079, which are NPBP in the same population. Satt489 is tightly linked to the *T* locus and to the *E1/e1* and *E7/e7* maturity loci and both *E1* and *T* have been implicated in chilling tolerance (Funatsuki and Ohnishi 2009). Satt489 is also tightly linked to Satt277 and Satt100, which are associated with QTLs for total seed weight (qCTTSW1), seed number (qCtsn2), single seed weight (aCTSSW1), and flowering time (qFLT1) following reproductive chilling (Funatsuki et al., 2005). The Satt227 to Satt291 genomic region and the Satt277 to Satt079 genomic regions are some distance apart but since QTLs are difficult to locate precisely these two neighboring genomic regions may both reflect a single genomic region affecting chilling tolerance.

Satt177 and Sat_115 on chromosome 8 (LG A2) are NPBP markers in both populations. They flank marker Sat_162, which is the peak marker for numerous co-locating QTLs affecting number of pods (PC5, PL5, and PL6), number of seeds per pod (SL5 and SL6), single seed weight (WL5), total seed weight (TL5 and TL6), number of nodes (NC6 and NL6), and maturity time (ML6) identified in the reproductive chilling study of Ikeda et al. (2009). Satt177 is also tightly linked to a sequence syntenic to marker Sat_313, linked to a QTL (HL6) affecting plant height under chilling stress (Ikeda et al., 2009). Furthermore, Satt177 is linked to a sequence syntenic to marker Sat_332, the peak marker for a QTL (WL6) reflecting single seed weight under chilling stress (Ikeda et al., 2009).

Satt192 on chromosome 12 (LG H) is a NPBP marker in both populations. It is tightly linked to the cytosolic ascorbate peroxidase locus *cAPX2*, the homolog of locus *cAPX1*, and a deficiency in *cAPX1* has been implicated in conferring chilling tolerance in soybean (Funatsuki et al., 2003). In the same genomic region are Sat_127, Satt635, and Satt666, which have been linked to a QTL (qCTTSW3) for total seed weight following reproductive chilling (Funatsuki et al., 2005) and also to two QTLs (NC6 and PC6) for number of nodes and number of pods, respectively, in nonstressed controls (Ikeda et al., 2009). Further down the chromosome, Satt253 gave a PBP correlation in X3015 and NPBP in X3050 as well as being

Table 1. Position in the soybean physical genome of simple sequence repeat (SSR) markers identified by bulked segregant analysis (BSA) as potentially affecting flowering time cold tolerance, relative to the positions of their orthologs, and to the positions of published quantitative trait locus (QTL), orthologs of published QTL, and classical loci associated with the trait.

Chromosome†	LG‡	Position of sequence (Mb)§	SSR marker	BSA result¶		Locus#	Published QTL, orthologs, or loci for chilling tolerance††	Genomic region is syntenic to	
				X3805	X3815			Marker	Chromosome
2	D1b	†	Satt216	PBP	bs				
2	D1b	6.307	Satt095	bs	NPBP				
2	D1b	7.282					Ortholog of Pub QTL	Sat_271	5
2	D1b	14.33					Ortholog of Pub QTL	Sat_321	10
6	C2	4.223	Satt227	NPBP	NPBP				
6	C2	4.947	Sat_062	bs	NPBP				
6	C2	6.524	Satt281	NPBP	bs				
6	C2	7.023	Satt520	NPBP	NPBP				
6	C2	7.228	Satt422	pm	NPBP				
6	C2	7.321	Satt291	PBP	pm				
6	C2	17.174	Satt277	pm	bs		Pub QTL		
6	C2	23.874	Satt489	PBP	bs				
6	C2	27.446	Satt289	nd	nd	T	Implicated locus		
6	C2	27.941	Satt134	nd	nd	E1	Implicated locus		
6	C2	30.668	Satt100	NPBP	bs		Pub QTL		
6	C2	37.34	Satt319	NPBP	ni	E7			
6	C2	43.951	Satt079	NPBP	bs				
8	A2	2.156					Ortholog of Pub QTL	Sat_332	1
8	A2	4.456					Ortholog of Pub QTL	Sat_313	13
8	A2	5.325	Satt177	NPBP	NPBP				
8	A2	8.279	Sat_162	nd	nd		Pub QTL		
8	A2	10.191	GMENOD2B	NPBP	ni				
8	A2	14.761	Sat_115	NPBP	NPBP				
8	A2	18.926	Satt437	pm	NPBP				
8	A2	20.512	Satt327	pm	NPBP				
12	H	0.308	Satt666	nd	nd		Pub QTL		
12	H	0.882	Satt635	nd	nd		Pub QTL		
12	H	4.265	Sat_127	bs	NPBP		Pub QTL		
12	H	4.52	Satt568	pm	NPBP				
12	H	5.39				cAPX2	Homolog of cAPX1	cAPX1	11
12	H	5.945	Satt192	NPBP	NPBP				
12	H	6.362	Satt442	bs	NPBP				
12	H	16.83						Satt079	6
12	H	18.644	Satt253	NPBP	PBP				
12	H	27.755	Satt279	bs	NPBP				
12	H	32.675					Ortholog of Pub QTL	AW756935	13
12	H	34.373					Ortholog of Pub QTL	Sat_197	13
12	H	35.108	Satt302	pm	NPBP				
12	H	36.046	Satt293	id	NPBP				
14	B2	8.21	Satt168	ni	PBP				
14	B2	8.643	Satt416	bs	NPBP				
14	B2	9.456						Satt227	6
14	B2	26.584						Satt281	6
14	B2	34.229	Satt070	NPBP	bs				
14	B2	45.427						Satt422	6
14	B2	46.706	Satt063	NPBP	pm				
14	B2	48.885						Sat_130	6
17	D2	6.053	Satt458	NPBP	NPBP				
17	D2	6.816					Ortholog of Pub QTL	Satt277	6

†Soybean (*Glycine max* (L.) Merr.) chromosomes numbered Gm01, etc., as per Soybase (http://www.soybase.org/; accessed 23 Apr. 2012). The physical location of Satt216 is not given in the *Glycine max* genome assembly version 1.01 (http://www.soybase.org/). However, BLAST analysis (Altschul et al., 1990) locates the Satt216 SSR primers to chromosome 2 (LG D1b) between Sat_351 and Sat_227, closely linked to Satt095. This is consistent with earlier recombination mapping, which placed Satt216 on the small LG W, which was subsequently joined to the top of large LG D1b, placing Satt216 and Satt095 approximately 1.2 cM apart (Cregan et al., 1999).

‡LG, linkage group.

§Genomic position based on *Glycine max* genome (assembly version 1.01) available at http://www.soybase.org/ (accessed 23 Apr. 2012).

¶The indicated SSR marker produced a perfect bulked segregant analysis banding pattern (PBP) or a near-perfect bulked segregant analysis banding pattern (NPBP) and correlation with the trait. Noninformative observations include parents are monomorphic (pm), bulks gave same banding pattern (bs), patterns were noninformative (ni) for other reasons, and incomplete data (id) or no data (nd) were obtained for that specific SSR.

#For classical loci, the physical location of the closest SSR marker is shown.

††Pub, published. Specific details and literature references are given in the text.

clustered with another NPBP marker (Satt279) in X3015. These two markers are tightly linked to sequences that are orthologous to AW756935 and Sat_197, respectively, markers that have been linked to a QTL (HL6) for plant height following reproductive chilling stress (Ikeda et al., 2009). It is not clear if these different regions on chromosome 12 represent one or multiple QTLs.

Satt168 on chromosome 14 (LG B2) is a PBP marker in X3815 and clustered with NPBP marker Satt416, but both are population specific. They are linked to a sequence syntenic to Satt227, a marker on chromosome 6 discussed previously.

Satt458 on chromosome 17 (LG D2) is adjacent to a sequence orthologous to Satt277, a marker on chromosome 6 discussed above.

Four of the above six chromosomal regions are related through homology. The region of interest on chromosome 14 contains sequences syntenic to Satt227, Satt281, and Satt422, markers that map near the top of the chromosome 6 region of interest. In addition, the region of interest on chromosome 17 contains a sequence syntenic to Satt277, which maps to the middle of the chromosome 6 region of interest. Finally, within the chromosome 12 region of interest is a sequence syntenic to Satt079, which maps to the bottom of the chromosome 6 region of interest. In contrast, the chromosome 2 and 8 regions appear to be unique.

The BSA technique was originally developed to mimic NILs and assist in searching for markers linked to a locus controlling a single gene trait (Michelmore et al., 1991). However, BSA has been used successfully to detect two loci affecting net blotch resistance in barley (*Hordeum vulgare* L.) plus a homologous region (Molnar et al., 2000). It was also successful at identifying multiple loci for crown rust resistance in oat (*Avena sativa* L.) (Wight et al., 2004), with co-selection of homologous genomic regions being observed. In the current study, BSA in two populations identified six genomic regions of interest, of which four are related by homology. The earlier cited BSA studies indicate that homologous regions may be co-selected during phenotypic selection both in building BSA bulks and in developing NILs and need not contain major genes directly affecting the trait. Alternatively, homologous regions may well contain homologous genes and the multiple versions may each be capable of influencing the phenotype. Therefore, the present results suggest that there are three to six chromosomal regions implicated in chilling tolerance in these half-sib populations. The large region on chromosome 6 may contain multiple loci since three other chromosomes show partial homology to it. Conversely, the small region on chromosome 17 may be retained during BSA purely due to homology. Three of the chromosomal regions (6, 8, and 12) align with loci or QTLs known to be associated with chilling tolerance and four (2, 8, 12, and 17) contain sequences syntenic with loci or QTLs known to be associated with chilling tolerance. Only the chromosome 14 region contains neither but is strongly homologous to the chromosome 6 region as discussed earlier. The BSA results focus and therefore considerably simplify future mapping, QTL analysis, or similar studies regarding reproductive chilling tolerance in this or related germplasm.

Table 2. Analysis of variance from field tests of selected cold tolerant and cold sensitive soybean lines grown in the field from 1998 to 2006, without 2005, at Ottawa, Canada.

Effect	Seed yield	Maturity	Seed protein	Seed oil	Seed weight	Plant height
Year	***	**	***	***	*	***
Replicate (year)	ns‡	ns	ns	ns	ns	**
Cross	ns	ns	**	ns	ns	***
Cold type	**	**	ns	ns	ns	***
Cross × cold type	*	**	**	**	*	*
Year × cross	ns	ns	ns	ns	ns	ns
Year × cold type	*	ns	ns	ns	ns	ns
Year × cross × cold type	ns	ns	ns	ns	**	ns

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

***Significant at the 0.001 probability level.

‡ns, not significant.

Field Tests of Selected Lines

A total of 12 CT and 13 CS lines from two crosses were tested in the field in Ottawa, Canada, over 8 yr. Initially the objective was to observe PP development and pod set on these lines in the field when a cold spell naturally occurred during flowering and to record seed yield and agronomic characteristics; however, no PPs were ever observed. In the ANOVA for seed yield (Table 2), the cross × cold type interaction parameter was significant for all observed traits so data is presented on a population–cold type basis (Table 3). In cross X3805, CT lines were 2 d later maturing and yielded about 9% more compared to CS lines while in cross X3815, CT lines were 9 d later maturing and yielded about 22% more compared to CS lines. Due to the differences in maturity it was not possible to directly compare the mean yield of CT and CS lines. In our experience, there is a 30 kg ha^{-1} d^{-1} relationship between seed yield and maturity in short-season soybean and when we used this value to adjust the CS yield upward, the CT lines outyielded CS lines by approximately 8%. As growing degree days accumulated in July decreased, CT lines yielded more than CS lines (Fig. 5) showing the importance of temperature during flowering. Other agronomic characters varied with cross and cold types but there were not consistent changes for seed protein and oil and seed weight (Table 3). Plant height differences paralleled seed yield differences. Most reports of seed yield following cold stress are based on single plant yield (Funatsuki et al., 2005; Ikeda et al., 2009; Kurosaki et al., 2003). Seed yield differences between a CT

Table 3. Mean seed yield and agronomic characteristics of selected cold tolerant (CT) and cold sensitive (CS) soybean lines grown in the field from 1998 to 2006, without 2005, at Ottawa, Canada.

Cross or line	Cold type	No. of lines	Seed yield	Maturity	Seed protein	Seed oil	Seed weight	Plant height
			kg ha^{-1}	days	g kg^{-1}	g kg^{-1}	g per 100	cm
X3805	CT	7	2794	115.7	396	218	17.7	74
X3805	CS	8	2552	113.6	401	216	17.3	69
X3815	CT	5	2831	119.4	409	215	16.8	71
X3815	CS	5	2316	110.7	404	219	17.7	59
SE			417	4.4	12	7	1.2	8.2
CV			16.2	3.9	2.9	3.2	7.1	11.9
9063	CT		3112	117.9	398	224	18.6	69
OT92-14	CT		2087	109.3	406	221	16.7	58
9007	CS		2730	113.4	396	218	15.5	69
86-CT	CT		2685	112.1	407	212	16.9	73
138-CS	CS		2465	112.2	413	204	17.9	69

Table 4. Mean seed yield and agronomic characteristics of lines resulting from cold tolerance (CT) × cold sensitive (CS) populations from trials at Ottawa in 2009 and at Morden and Ottawa in 2010.

Cross†	Cross type	No. of lines	Seed yield	Maturity	Seed protein	Seed oil	Seed weight	Plant height
			kg ha^{-1}	days	g kg^{-1}	g kg^{-1}	g per 100	cm
X5105	CT × CT	24	3982 a‡	121.9 b	396 c	209 a	17.7 a	84 a
X5106	CT × CS	21	3871 b	121.2 b	402 ab	206 b	17.8 a	87 a
X5107	CS × CT	23	3792 b	122.9 a	400 b	207 ab	17.9 a	85 a
X5108	CS × CS	22	3698 c	122.3 ab	405 a	200 c	17.9 a	87 a
SE			259	2.9	9	6	1.3	9
CV			6.8	2.4	2.2	2.9	7.1	10.4

†Cross pedigrees: X5105 (86-CT × OT99-8), X5106 (86-CT × 9007), X5107 (138-CS × OT99-8), and X5108 (138-CS × 9007).

‡Values followed be a different letter are significantly different, LSD (p = 0.05).

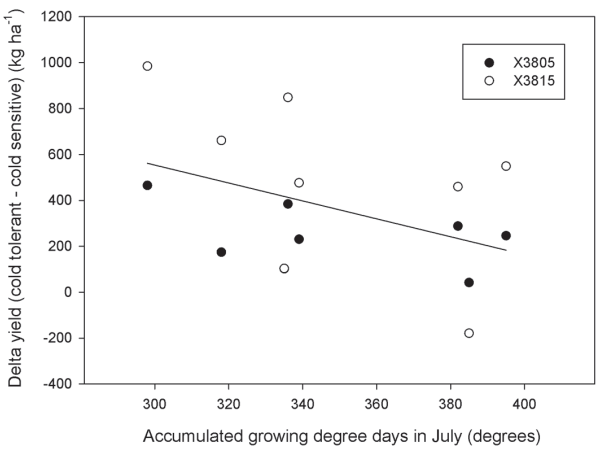

Figure 5. The seed yield difference between cold tolerant and cold sensitive lines from two crosses (X3805 [9063 × 9007] and X3815 [OT92-14 × 9007]) compared to accumulated growing degree days in July over eight growing seasons at Ottawa, Canada.

and CS cultivar have been reported using single-row plots in the field in 1 of 2 yr (Funatsuki et al., 2004), but larger scale yield trials have not been reported.

Previous studies have shown a relationship between pubescence color and seed yield where tawny lines yielded more in cool seasons than gray lines (Morrison et al., 1994, 1997) or tawny lines were more chilling tolerant compared to gray lines (Toda et al., 2011). In our two crosses, the CT parents had tawny pubescence and CS parent had gray pubescence; however, the selected CT progeny (four gray and eight tawny) and CS progeny (five gray and eight tawny) were mixed for pubescence color. Using BSA one genomic region was identified on chromosome 6 (containing the *T* locus), but other regions on other chromosomes were also identified (Table 1).

To reduce maturity differences, a second set of crosses were made using a CT (86-CT) and CS (138-CS) line with similar maturity both derived from cross X3805 (Table 3). Each line was crossed to 9007, the original CS parent, and to OT99-8, an unreleased early maturing line with cold tolerance. Resulting randomly derived RILs with early maturity were field tested. In tests in three environments, mean maturities were similar (Table 4). Although the mean yield of lines from each population was confounded with the parents used, the CT × CT lines yielded on average 8% more than the CS × CS lines while the lines from CS × CT parents yielded 5 and 3% more than the CS × CS lines (Table 4). The crosses did not vary for seed weight or plant height while CT lines had lower seed protein and higher seed oil compared to the CS lines (Table 4).

In summary, cold screening of soybean lines for a 3 or 6 wk period following the onset of flowering was optimized by scoring plants 6 wk following the conclusion of the cold treatment. Lines characterized as CT yielded about 8% more than CS lines in the field even in the absence of visible cold damage symptoms.

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