Development of AFLP and STS markers linked to a waterlogging tolerance in Korean soybean landraces

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Abstract

Among the 400 soybean (*Glycine max*) landraces, we selected 3 tolerant (KAS150-9, KAS160-15, and KAS170-9) and 3 susceptible lines (KAS160-14, KAS160-20, and KAS201-6-1) by the survival percentage and injury scores. Susceptible lines showed decrease in chlorophyll content and increase in glucose and malondialdehyde (MDA) contents under waterlogging stress, while tolerant lines did not change significantly. For AFLP analysis, 8 *Eco*RI (+3) and 8 *Mse*I (+3) primers used in 32 primer combinations generated a total of 2 566 bands with a mean of 80 bands per primer combination, of which 1 117 (43.5 %) were clearly polymorphic between the tolerant and susceptible lines. A genetic similarity coefficient, based on cluster analysis using an unweighted pair grouping method of average (UPGMA), was 0.79 for the tolerant group, while the susceptible landraces were genetically less related, with a genetic similarity coefficient of 0.17. The 10 reproducible polymorphic PCR products present in the 3 tolerant or susceptible lines were sequenced and converted into sequence tagged site (STS) markers. These STS primer sets were designated GmWT01-GmWT06 and GmWS01-GmWS04. Two STS primer sets, GmWT06 and GmWS02, generated a single monomorphic PCR product identical in size to the original AFLP fragments. For the broad application of these STS markers in marker-assisted selection (MAS) for soybean genotypes tolerant to waterlogging stress, two developed STS markers are being evaluated with putative waterlogging tolerant mutant lines induced by γ-radiation in soybean mutation breeding programs.

Additional key words: chlorophyll, *Glycine max*, malondialdehyde, MAS, UPGMA.

Introduction

Soybean (*Glycine max* L. Merr), one of the important food legumes, is generally susceptible to waterlogging stress (Linkermer *et al.* 1998, Oosterhuis *et al.* 1990). The main reason for the detrimental effects of excess soil moisture is the lowered oxygen concentration in the gaseous phase of a soil. The most common symptom of waterlogging stress is leaf chlorosis, which is followed by necrosis, stunting, defoliation, and plant death (Cornelious *et al.* 2005). The causes of yield loss likely arise from reduced root growth, nodulation, nitrogen fixation, photosynthesis, biomass accumulation, stomatal conductance, *etc*. (Oosterhuis *et al.* 1990, Reyna *et al.* 2003, Ortuño *et al*. 2007).

The amplified fragment length polymorphism (AFLP) is a powerful tool for tagging genes or quantitative trait loci (QTLs) of interest in plants. The AFLP method is capable of generating larger numbers of molecular markers than restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), or simple sequence repeat (SSR) (Vos *et al.* 1995, Powell *et al.* 1996, Garcia *et al.* 2004). In case where AFLP bands contain repeated sequence, the ability to clone and sequence specific bands provides the opportunity to convert them into sequence tagged sites (STS) for further genetic analysis (Kim *et al.* 2004). In the case of resistance genes to specific stress, it is possible to design primers, usually 18 - 25 bp long, for PCR reactions that will amplify the AFLP alleles that segregate with the resistance gene. Plant breeders can use these primers to screen plant populations to find those plants that generate

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Abbreviations: AFLP - amplified fragment length polymorphism; MDA - malondialdehyde; STS - sequence tagged site.
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PCR amplification products. For efficient marker assisted selection (MAS), conversion of the linked markers into STS allows a large number of plants to be genotyped by a PCR assay (Meksem *et al.* 1995). MAS is most efficient when there is a tight linkage between the marker and the trait of interest (Kelly 1995). This approach has been demonstrated to be feasible in many crops such as barley (Decousset *et al.* 2000) and soybean (Meksem *et al.* 2001). In our study, markers for waterlogging tolerance may also offer the additional advantage of allowing selection for tolerance in the absence of the cultivation on a paddy field under intense rainfall or excessive irrigation.

We selected 3 tolerant (KAS150-9, KAS160-15, and KAS170-9) and 3 susceptible lines (KAS160-14, KAS160-20, and KAS201-6-1) among 400 Korean soybean landraces with morphological criteria such as the survival percentage and injury scores. The objectives of this study were to identify AFLP markers closely associated with waterlogging tolerance from the selected lines, and ultimately to develop sequence-specific PCR markers from these AFLP markers for easy and stable identification of water logging tolerant landraces or mutants in an early generation.

Materials and methods

Plants and waterlogging treatment: The experiment was conducted at the Breeding Research Farm of Korea Atomic Energy Research Institute during the 2005 season. Four hundred Korean soybean landraces were grown for screening of water logging tolerance in a dried paddy field. The experimental design was a split plot arrangement with three replications, and each plot (3 × 1.8 m) consisted of three rows with planting density of 60 × 10 cm. Soybean landraces were sown on earthen levees 15 cm above soil surface to prevent natural flooding by heavy rain. Flooding treatment was conducted at the early reproductive stage (R2) for 10 d from 29 July to 8 August until the water level reached 3 cm above the soil surface. Water was maintained at this level until moderate canopy chlorosis and necrosis appeared about 10 to 12 d later. Then, water was allowed to drain from each plot. Conventional crop management, methods such as weeding, disease and insect control, were followed during the growing period. Three tolerant [KAS150-9(T1), KAS170-9(T2), KAS160-15(T3)] and three susceptible [KAS160-14(S1), KAS160-20(S2), KAS201-6-1(S3)] lines were selected by the percentage of survival and germination and by a visual rating scale of 0 to 4 (0 - no injury of survival plants, 1 - < 30 % chlorosis, 2 - < 50 % chlorosis, 3 - < 80 % dead, 4 - > 95 % dead plants). These lines were confirmed for water logging tolerance in a randomized complete block design with three replications using pots (15 cm of diameter) and stainless container with drain facility on a bed of glasshouse. Selected lines were analyzed for biochemical measurement such as chlorophyll, malondialdehyde (MDA), and glucose contents and screened with AFLP for development of marker linked to waterlogging tolerance.

Biochemical measurements: Samples for analyses were taken from plants in the mid row of each tolerant or susceptible line at 0, 2, and 4 d after waterlogging treatment. All of the analyses were performed on the third and fourth leaves from the top of the plant. Chlorophyll was extracted in acetone according to the method of Lichtenthaler (1987), measured with a spectrophotometer *Uvikon 923* (*Bio-Tek Instruments*, VT, USA) and the content was calculated according to Arnon (1949). Soluble sugars were extracted from leaf discs, with 80 % (v/v) ethanol at 80 °C for 20 min. The extracts were centrifuged at 10 000 *g* for 10 min. The supernatant (1 cm^3) was mixed with 2 cm^3 of 4 % phenol and 5 cm^3 of concentrated sulfuric acid. The absorbance was read at 490 nm. The level of lipid peroxidation was estimated according to the method of El-Moshaty *et al.* (1993) by measuring the concentration of MDA by reaction with thiobarbituric acid (TBA). Fresh leaf samples (0.5 g) were ground with 5 cm^3 of 0.1 % trichloroacetic acid (TCA). The samples were centrifuged at 10 000 *g* for 15 min. The supernatant fraction was mixed with 4 cm^3 of 20 % (m/v) TCA containing 0.5 % (m/v) TBA. The mixture was incubated at 95 °C for 30 min and then quickly cooled in an ice bath for 15 min. After centrifugation at 10 000 *g* for 10 min, the absorbance of the extract was read at 532 nm. A correction for non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. Due to the limited specificity of the method, the concentration of TBA reactive species (TBARS) was calculated by using the coefficient of absorbance of 155 mM cm^{-1}.

AFLP analysis: DNA was isolated from 0.1 g of fresh leaf tissue using a plant genomic DNA extraction miniprep system (*Boehringer Mannheim*, Mannheim, Germany). AFLP analysis was conducted using AFLP analysis system II according to the manufacturer's instructions (*Invitrogen*, Carlsbad, USA). Approximately 250 ng genomic DNA from each sample was restricted with 3 U *Eco*RI and *Mse*I followed by the *Eco*RI/*Mse*I adapter ligation reaction. After diluting the ligated DNA 1/10 in TE buffer, pre-amplification was performed using a *PTC-100* thermal controller (*MJ Research*, Waltham, USA) following the cycle profile: 30 s DNA denaturation step at 94 °C, 60 s annealing step at 56 °C, and 60 s extension step at 72 °C. Pre-amplified DNA was diluted to 1/50 and the prepared DNA was used as a template for selective amplification. Thirty-two *Eco*RI/*Mse*I primer combinations were used for selective amplification

(AFLP small genome primer kit; *Gibco BRL*, Rockville, USA). The selective primers for AFLP analysis are used 8 *Eco*RI + 3 primers (*Eco*RI + AAC, AAG, ACA, ACT, ACC, ACG, AGC, and AGG) and *Mse*I + 3 primers (*Mse*I + CAA, CAC, CAG, CAT, CTA, CTC, CTG, and CTT). The PCR program for selective amplification was 4 min at 94 °C for pre-heating, followed by 10 cycles of 60 s at 94 °C, 60 s at 65 °C, and 90 s at 72 °C, the annealing temperature being reduced by 1 °C at each cycle, followed by 33 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. PCR products were analyzed on 1.5 % agarose gels. Selective amplified products were fractionated on 6 % denaturing polyacrylamide gels, and stained using Silver Sequence DNA Staining reagents (*Promega*, Madison, USA) according to the manufacturer's instructions.

Polymorphism was scored as presence or absence of bands in each line and data were analyzed for clustering using the *NTSYSpc* version *2.0* (*Exeter Software*, NY, USA). The results were converted into a similarity matrix utilizing the SIMQUAL (similarity for qualitative data) method. The similarity coefficient was used for cluster analysis following the unweighted pair grouping method of averages (UPGMA). The resulting clusters were represented in the form of a dendrogram.

Cloning of AFLP fragments and conversion into STS markers: A polymorphic AFLP fragment of interest was excised and eluted in 0.05 cm^3 of sterile water by heating at 100 °C for 15 min. The tubes were centrifuged briefly and the supernatant was transferred to a new tube. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.6) and 3 volumes of 100 % ethanol. The elute of 0.01 cm^3 was re-amplified using the same primer combinations. PCR was carried out in a reaction mixture containing 0.5 μM *Eco*RI primer, 4.5 μM *Mse*I primer, 2.5 mM dNTPs, 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and 2U *Taq* polymerase. The PCR profile was: 30 cycles with 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C, and then finally incubation at 72 °C for 4 min. Amplified products were separated on 1.5 % agarose gels, and eluted using the nucleic acid purification kit (*Bioneer*, Daejeon, Korea). Purified DNA fragments were ligated into the vector *pGEM-T Easy* (*Promega*). Inserted DNA was amplified using either the T3 or the SP6 promoter primer (*Promega*) with the big dye terminator cycle sequencing ready reaction kit (*Perkin Elmer*, Foster City, USA) according to the manufacturer's instructions. Sequencing was performed on the *ABI 3730* genetic analyzer (*PE-ABI*, Foster City, USA).

The forward and reverse primers were designed from the end sequences of the AFLP fragments. A PCR reaction mixture was optimized according to the respective primer annealing temperatures (Table 2) and the results were separated in 2 % (m/v) agarose gel with 0.5 × TBE running buffer and visualized by ethidium bromide staining.

Statistics: We performed a two-factor *ANOVA* (landrace and flooding duration, as main effects) in order to observe each individual landrace, flooding duration, and interaction of landrace and flooding duration. Further the least significant differences ($LSD_{0.05}$) were calculated using the *SAS* program (*SAS Institute*, Cary, NC, USA).

Results and discussion

We selected 3 tolerant [KAS150-9(T1), KAS170-9(T2), and KAS160-15(T3)] and 3 susceptible [KAS160-14(S1), KAS160-20(S2), and KAS201-6-1(S3)] lines by their germination and survival rates and by the injury score. In the tolerant landraces, the germination and survival percentage was 88.2 and 92.8 % in average, while the susceptible lines showed 65.5 and 0 %, respectively. This result was observed by glasshouse experiments in a randomized complete block design (data not shown). These landraces were further analyzed for evaluating biochemical measurements and development of genetic markers by AFLP analysis.

Waterlogging stress reduced total chlorophyll (Chl) contents both in tolerant and susceptible lines as stress duration increased from 0 to 4 d (Fig. 1*A*). At 2 d of waterlogging, there were no differences in Chl contents between tolerant (T) and susceptible (S) lines except for S3. The *ANOVA* analysis showed that there was a significant reducing effect on Chl contents by the waterlogging duration, but effects of the landrace and interactions between the waterlogging duration and landrace were not significant. Similar finding of decreasing Chl content were reported in wheat by Makhmudov (1983), who postulated inhibition of precursor biosynthesis resulting in reduced total Chl content. The reduction of Chl caused low photosynthesis and low relative growth rate and biomass accumulation (Chen *et al.* 2002). Seong *et al.* (2000) reported that stem and leaf growth of soybean were significantly inhibited by prolonged excessive soil moisture at the R2 growth stage, and in the end, yield-related characters such as pod number, seed number and seed mass were highly reduced. Kozlowski (1984) suggested that the initial effect of waterlogging on photosynthesis was not caused by leaf Chl loss but by partial stomatal closure. It has been reported that many wetland species initially closed stomata in response to soil flooding.

Leaf glucose content increased with the increase of waterlogging duration in all examined landraces. Increase of the glucose content in the S lines was greater than in the T lines (Fig. 1*B*). At 4 d of waterlogging, leaf glucose content in the S lines increased by 37 to 73 % compared

to day 0. However, the T lines had no significantly increased glucose except for T1. The *ANOVA* analysis showed that there was a significant effect of the landrace and waterlogging duration on the glucose content in leaves, but no significant interaction was detected between two main effects. Under flooding stress, the inhibition of photosynthesis was reported to be accompanied by increased concentrations of soluble sugar and starch in leaves of *Quercus alba* (Gravatt and Kirby 1998). Similar results have been reported in

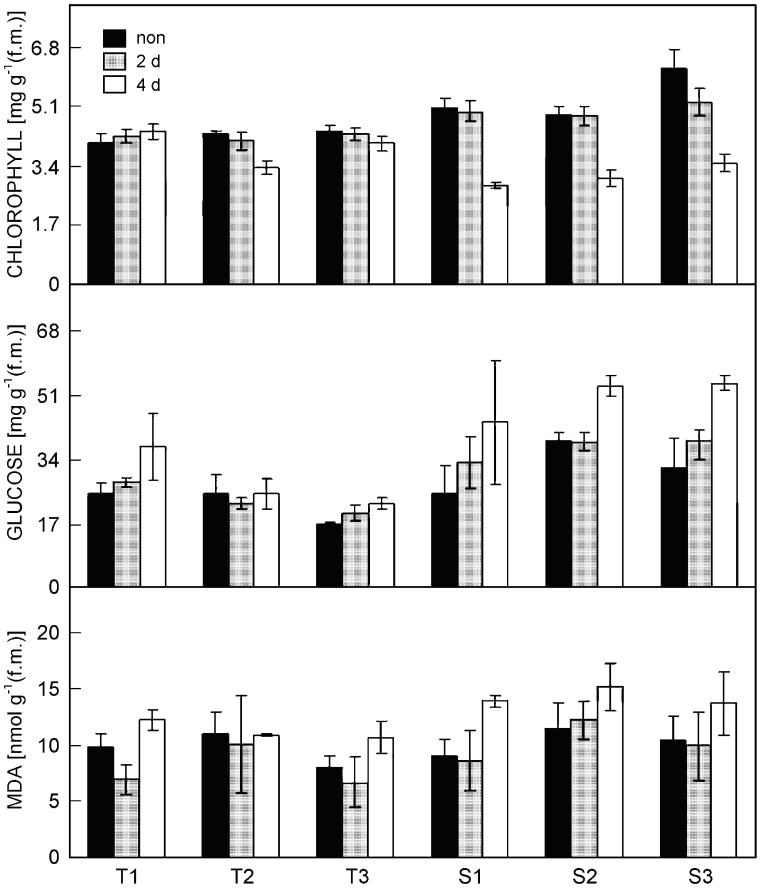

Fig. 1. Changes of total chlorophyll, glucose, and MDA contents during water logging treatment in the 3 tolerant (T) and 3 susceptible (S) lines.

Table 1. Number of polymorphic AFLP bands observed using 32 AFLP primers combinations (2566 total bands, 1117 polymorphic).

Primer combinations	Total bands	Polymorphic bands [%]	Primer combinations	Total bands	Polymorphic bands [%]
E-ACT/*M*-CTT	53	30 (56.6)	*E*-AAC/*M*-CAT	107	51 (47.7)
E-AAC/*M*-CAA	59	28 (47.5)	*E*-ACA/*M*-CAG	124	60 (48.4)
E-ACA/*M*-CAA	69	51 (73.9)	*E*-ACC/*M*-CAG	91	46 (50.5)
E-ACT/*M*-CAA	94	31 (33.0)	*E*-AGC/*M*-CAG	148	74 (50.0)
E-ACG/*M*-CAA	100	60 (60.0)	*E*-AGG/*M*-CAG	91	43 (47.3)
E-AGC/*M*-CAA	101	46 (45.5)	*E*-ACC/*M*-CAA	101	35 (34.7)
E-AGG/*M*-CAA	116	39 (33.6)	*E*-AAG/*M*-CAA	116	44 (37.9)
E-AAG/*M*-CAC	52	15 (28.8)	*E*-ACG/*M*-CAG	54	28 (51.9)
E-ACT/*M*-CAC	90	42 (46.7)	*E*-ACT/*M*-CAG	105	48 (45.7)
E-ACC/*M*-CAC	94	30 (31.9)	*E*-ACT/*M*-CTA	146	76 (52.1)
E-ACG/*M*-CAC	53	22 (41.5)	*E*-ACT/*M*-CAT	81	21 (25.9)
E-AGC/*M*-CAC	83	31 (37.3)	*E*-AGC/*M*-CTC	17	2 (11.8)
E-AGG/*M*-CAC	55	21 (38.2)	*E*-ACA/*M*-CTG	21	11 (52.4)
E-AAC/*M*-CAC	69	27 (39.1)	*E*-AAC/*M*-CTG	80	46 (57.5)
E-AAG/*M*-CAC	62	16 (25.8)	*E*-AAG/*M*-CTG	22	6 (27.3)
E-ACA/*M*-CAC	78	23 (29.5)	*E*-ACC/*M*-CTG	34	14 (41.2)

sunflower (Wample and Davies 1983) and winter rape (Leul and Zhou 1999). This means that delivery of photosynthates from leaves to roots was inhibited under flooding stress, resulting in sugar accumulation in leaves of flooded plants. The inhibition of the sugar translocation might be much greater than the decrease in net photosynthesis.

Waterlogging treatment increased contents of malondialdehyde (MDA) by 31 to 54 % in leaves of the three S lines. But the T lines showed no difference in the MDA content. On day 2 after the waterlogging treatment, the MDA contents were not increased in the T and S lines. But 4 d after the treatment, the S lines showed significantly increased MDA contents compared to the T lines (Fig. 1*C*). In the *ANOVA* analysis, there was a significant effect of the waterlogging duration. The MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero 1990). Oxidative damage in plants is involved in processes like leaf senescence or wounding (Thompson *et al.* 1987), and has been used to determine susceptibility to water stress (Moran *et al.* 1994) and oxygen stress (Hunter *et al.* 1983). In this study, we observed that the content of MDA increased in the S lines which indicated that oxidative damage in the S lines was higher than in the T lines. A rise in the MDA content was reported in susceptible species such as *Brassica napus* (Leul and Zhou 1999) and *Iris germanica* (Hunter *et al.* 1983). In contrast, the MDA content remained the same in *Iris pseudacorus*, an anoxia tolerant wetland species (Hunter *et al.* 1983). Anoxia-tolerant species may be better protected against oxygen free radicals by the accumulation of antioxidants (Larson 1988).

To identify the variation frequency among the waterlogging tolerant or susceptible landraces, and to develop specific markers for waterlogging tolerance, AFLP analysis was performed with the selected T and S lines. The eight *Eco*RI (*E*) (+3) and *Mse*I (*M*) (+3) primers gave 32 different primer combinations with different selective nuclotides. The 32 primers produced 2 566 bands with an average of 80.2 per primer combination, of which 1 117 (43.5 %) were polymorphic (Table 1). The largest number of polymorphic bands (76) was produced with primer combination *E*-ACT/*M*-CTA, and the least number of polymorphic bands (2) was detected using primer combination *E*-AGC/*M*-CTC. These combinations, with three and three selective nucleotide extensions on the *Eco*RI and *Mse*I primers, respectively, were sufficient to produce the required number of bands. Figs. 3*A* and *C* give examples of the DNA fingerprints and the DNA polymorphisms obtained with 2 of the primer combinations tested (*E*-ACT/*M*-CAT and *E*-ACA/*M*-CAC). These AFLPs were subsequently converted into STS markers.

Genetic similarity (GS) coefficients for 15 possible pairs among the T and S lines, based on the above 1 117 polymorphic bands, ranged from 0.8732 in a solitary of landarces (between the T1 and T3) to 0.2536 in a solitary of landraces (between the T lines and S3). The dendrogram generated with the UPGMA cluster analysis of the genetic similarity coefficients matrix on the basis of these GS values revealed two clusters: cluster I containing three 3 waterlogging tolerant landraces and cluster II containing 3 waterlogging susceptible landraces (Fig. 2). The tolerant lines were grouped by genetic similarity value of 0.79, while the susceptible landraces were genetically less related.

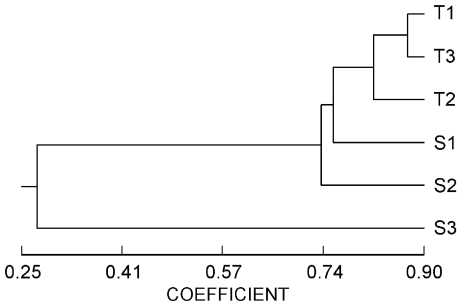

Fig. 2. Dendrogram of the 3 tolerant (T) and susceptible (S) soybean landraces revealed by unweighted pair grouping method of averages (UPGMA) cluster analysis of amplified fragment length polymorphism (AFLP)-based genetic similarity (UN 1 coefficient) estimates using 1 117 AFLP polymorphic bands obtained using 32 primer pairs. T1 - KAS150-9, T2 - KAS170-9, T3 - KAS160-15, S1 - KAS160-14, S2 - KAS160-20 and S3 - KAS201-6-1.

The 26 polymorphic PCR products present only in the T or S lines were cloned and sequenced in order to convert these AFLP markers into sequence tagged site (STS) markers. Sequence comparisons of the 26 AFLP fragments with non-redundant accessions in the database at the NCBI using *BLAST* program revealed no significant homology with nucleotide sequences registered in the databases. For STS analysis, primers for 10 out of the 26 sequenced products were designed from sequences obtained from the *Eco*RI adapter at one end and the *Mse*I adapter at the other end. The length of the sequence-specific primers, designated as GmWT-01 to GmWT-06 designed from the T line-specific PCR products (AFLPs present only in the T lines) and GmWS-01 to GmWS-04 designed from the S line-specific PCR products (AFLPs present only in the S lines), varied from 21 bp to 24 bp. The sequence, annealing temperature, and PCR conditions of each primer set are listed in Table 2.

To determine whether or not AFLPs were successfully converted into STS markers, the designed STS primer sets were tested against genomic DNA from the T and S lines. PCR conditions for each STS marker were varied to optimize DNA banding patterns of the expected size. Yu and Pauls (1994) proposed that the time for each step in the PCR program might need to be optimized for differently sized primers and/or DNA templates from different species. In the PCR program, altering the duration of each step, and the number of cycles, revealed different banding patterns. The two STS primer sets, GmWT-06 and GmWS-02, generated a single polymorphic PCR product identical to the original AFLP

products in size (Fig. 3*B*,*D*). But the other 8 primer sets revealed DNA banding patterns with more than two bands in addition to the fragment of the expected size. These banding patterns were caused by either slippage events during the PCR amplfication process (Tautz 1990), terminal transferase activity of the enzyme, and/or incomplete extension by polymerase (Love *et al.* 1990).

In order to verify whether the polymorphic fragments from GmWT-06 and GmWS-02 primer pairs were specific for waterlogging tolerance, further PCR analyses were conducted. We used 4 Korean soybean cultivars (cvs. Iksannnamulkong, Taegwangkong, Myungjuna-mulkong, and Jinpumkong-1) and 4 landraces (KAS 150-22, KAS200-17-2, KAS 130-5 and KAS174-3).

Table 2. Sequences of STS primers derived from AFLP polymorphic DNA fragments. Designed STS primer sets specific to the tolerant or susceptible lines. [b] - Denaturation time [s], annealing time [s], extension time [s], and the number of cycles used were shown for each primer.

T/S	AFLP combination	Name	Sequence (5' → 3')	Annealing temp. [°C]	Size [bp]	PCR condition[b]
T	*E*-AGC/*M*-CAC	GmWT01	GCTGCACACACTTTCCTTTTGTGC CAGGTATACAAAGACGCATTGC	60	193	30, 30, 30, 33
	E-AGG/*M*-CAA	GmWT02	GGGTGATGAATGTCAAGCCAC ATGAATGATGTGTACTTGGAAG	57	92	30, 30, 30, 35
	E-ACC/*M*-CAC	GmWT03	GACAGGGTCCGAGATCAAATATG CACACTAGTTTTCATAAAAAC	55	79	30, 30, 30, 40
	E-AGG/*M*-CAA	GmWT04	CATGACATGCTGATTCTATAAC CAAGATAAGTCCTGAATTGGTA	54	195	30, 30, 30, 40
	E-AGG/*M*-CAA	GmWT05	GAAGCAGGACATTGATACTTTTG CAACCTTTGAGATATCTTGAAACC	57	157	30, 30, 30, 40
	E-ACT/*M*-CAA	GmWT06	CACCCACTAACTTAGGGTCTTG GTCCTTCAAGATTGTGAGCAAC	62	171	30, 30, 30, 28
S	*E*-AGC/*M*-CAC	GmWS01	CCATCCGCAAGTAGCATATCCTAG CATGAACAACACAGGGACAGCTG	62.	89	30, 30, 30, 30
	E-ACA/*M*-CAC	GmWS02	CTTACGAAAGCGCCTCAACCTTG CGTAATTCATAGGGTTTTGTG	57	377	30, 30, 30, 35
	E-ACG/*M*-CAA	GmWS03	CTCATCAAAGCCCTCTCTGATTCC CAACAACGTCAATATAATTACCTG	58	145	30, 30, 30, 40
	E-AGG/*M*-CAA	GmWS04	CAGATGAATTTCGGGCAGTATCC CAGCAACAAACCTGAGTAGGC	58	222	30, 30, 30, 35

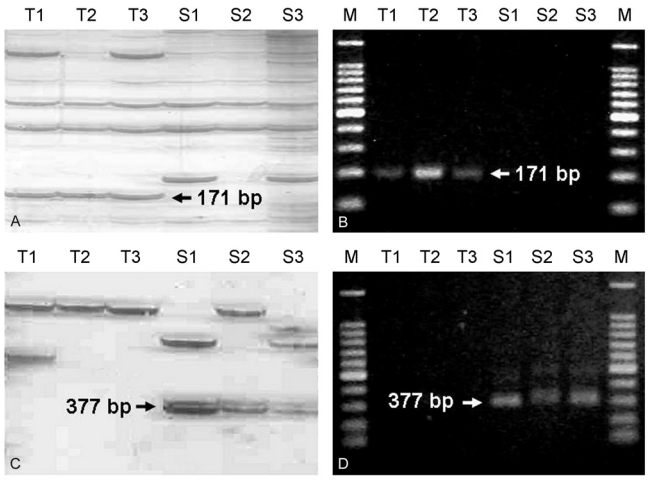

Fig. 3. Variation in AFLP patterns between the waterlogging tolerant (T) and susceptible (S) soybean landraces and development of STS markers. Silver-stained polyacrylamide gels showing T lines-specific (*E*-ACT/*M*-CAT) or S lines-specific (*E*-ACA/*M*-CAC) amplified fragments detected in the T or S lines, respectively. The AFLP second-round primer conditions (*E*-ACT/*M*-CAT) (*A*) and (*E*-ACA/*M*-CAC) (*C*) generated polymorphic bands. *Arrows* indicate putative waterlogging tolerant- or susceptible-specific bands. The polymorphic AFLPs were converted into STS primer sets. Two STS primer sets, GmWT-06 (*B*) and GmWS-02 (*D*), amplified a single band of 171 bp and 377 bp, respectively. GmWT-06 and GmWS-02 were converted from the polymorphic AFLPs generated by E-ACT/M-CAT (*A*) and E-ACA/M-CAC (*C*) second-round primer sets, respectively. M indicates 100 bp DNA size marker. Amplification conditions for each STS marker are described in Table 2.

Amplified DNA products of about 171 bp were generated with the GmWT-06 primer set in Iksannnamulkong, Taegwangkong, KAS150-22, and KAS200-17-2 (Fig. 4*A*). Primer set GmWS-02 amplified a fragment of expected size 377 bp using template from Myungjunamulkong, Jinpumkong-1, KAS130-5, and KAS174-3 (Fig. 4*B*). Cvs. Iksannnamulkong and Taegwangkong were cultivated in the paddy field as waterlogging tolerant cultivars on the recommendation of the Rural Development Administration in Korea. On the other hand, cvs. Myungjunamulkong and Jinpumkong-1 were reported as waterlogging susceptible lines. In our study, KAS 150-22 and KAS200-17-2, showed injury score 1, while KAS 130-5 and KAS174-3 had injury score 4.

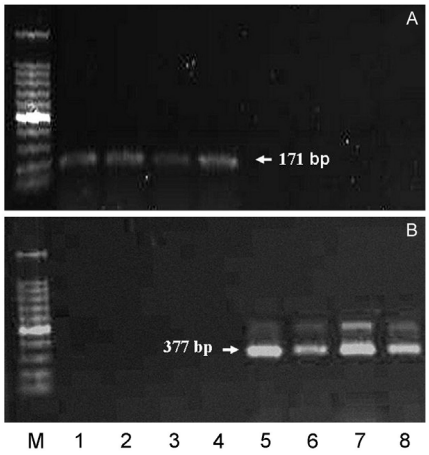

Fig. 4. PCR amplification products generated in 8 soybean lines using STS marker sets GmWT-06 (*A*) and GmWS-02 (*B*). *Lane* M - 100 bp DNA size marker, *lane* 1 - Iksannnamulkong, *lane* 2 - Taegwangkong, *lane* 3 - KAS150-22, *lane* 4 - KAS200-17-2, *lane* 5 - Myungjunamulkong, *lane* 6 - Jinpumkong-1, *lane* 7 - KAS130-5, *lane* 8 - KAS174-3.

AFLP is a PCR based multi-locus fingerprinting technique, which efficiently identifies DNA polymorphisms without prior information on the DNA sequence of the organisms (Brugmans *et al.* 2003). Unless new DNA sequencing technologies that combine reliability and cost effectiveness are invented, AFLP will continue to be used for rapid and efficient genome-wide allele scoring and linkage mapping (Meksem *et al.* 2001). However, the AFLP analysis is a relatively complex procedure, thus, for large-scale screening of breeding materials in germplasm improvement programs, AFLP markers are often converted to reliable codominant STS markers which are easy to handle (Murakami *et al.* 2005). However, it is also usual that AFLP markers are not necessarily easily converted to STS markers. We could also convert only 2 primer pairs (GmWT06 and GmWs02) out of 10 analyzed AFLPs into STS markers. Shan *et al.* (1999) conducted a conversion of chromosome-specific AFLP markers to STS markers, 6 out of the 26 primer sets derived from the AFLP marker amplified a fragment from the expected chromosome in wheat and barley. Seo *et al.* (2001) conducted a conversion of AFLP markers to STS markers in wheat, but only 1 (SJ07) out of the 12 primer pairs derived from the AFLP marker sequence data could serve as a potential 2RL-specific markers.

The identification of STS markers linked to waterlogging tolerance in soybean will be useful for marker assisted selection (MAS) to expedite the development of high-yielding waterlogging tolerant soybean cultivars. The identification of markers associated with waterlogging tolerance will allow breeder to select lines with the waterlogging tolerant trait at the DNA level.

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Brugmans, B., Van Der Hulst, R.G., Visser, R.G., Lindhout, P., Van Eck, H.J.: A new and versatile method for the successful conversion of AFLP markers into simple single locus markers. - Nucl. Acids Res. **31** (Suppl. 10): e55, 2003.

Chen, H., Qualls, R.G., Miller, G.C.: Adaptive responses of *Lepidium latifolium* to soil flooding: biomass allocation, adventitious rooting, aerenchyma formation and ethylene production. - Environ. exp. Bot. **48**: 119-128, 2002.

Cornelious, B., Chen, P., Chen, Y., De Leon, N., Shannon, J.G., Wang, D.: Identification of QTLs underlying water-logging tolerance in soybean. - Mol. Breed. **16**: 103-112, 2005.

Decousset, I., Griffiths, S., Dunford, R.P., Pratchett, N., Laurie, D.A.: Development of STS markers closely linked to the PPd-H1 photoperiod response gene of barley (*Hordeum vulgare* L.). - Theor. appl. Genet. **101**: 1202-1206, 2000.

El-Moshaty, F.I.B., Pike, S.M., Novacky, A.J., Sehgal, O.P.: Lipid peroxidation and superoxide production in cowpea (*Vigna unguiculata*) leaves infected with tobacco ringspot virus or southern bean mosaic virus. - Physiol. mol. Plant Pathol. **43**: 109-119, 1993.

Garcia, A.A.F., Benchimo, L.L., Barbosa, A.M.M., Geraldi, I.O., Souza, C.L., Jr., De Souza, A.P.: Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. - Genet. mol. Biol. **27**: 579-588, 2004.

Gravatt, D.A., Kirby, C.J.: Patterns of photosynthesis and starch allocation in seedlings of four bottomland hardwood tree species subjected to flooding. - Tree Physiol. **18**: 411-417, 1998.

Hunter, M.I.S., Hetherington, A.M., Crawford, R.M.M.: Lipid peroxidation a factor in anoxia intolerance in *Iris* species. - Phytochemistry **22**: 1145-1147, 1983.

Janero, D.R.: Malondialdehyde and thiobabituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. - Free Radicals Biol. Med. **9**: 515-540, 1990.

Kelly, J.D.: Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. - HortScience **30**: 461-465, 1995.

Kim, D.S., Lee, I.S., Jang, C.S., Kang, S.Y., Song, H.S., Lee,

Y.I., Seo, Y.W.: Development of AFLP-derived STS markers for the selection of 5-methyltryptophan-resistant rice mutants. - Plant Cell Rep. **23**: 71-80, 2004.

Kozlowski, T.T.: Plant responses to flooding of soil. - BioScience **34**: 162-167, 1984.

Larson, A.R.: The antioxidants of higher plants. - Phytochemistry **27**: 969-978, 1988.

Leul, M., Zhou, W.J.: Alleviation of waterlogging damage in winter rape by uniconazole application: effects on enzyme activity, lipid peroxidation, and membrane integrity. - Plant Growth Regul. **18**: 9-14, 1999.

Lichtenthaler, H.K.: Functional Organization of Carotenoids and Prenylquinones in the Photosynthetic Membrane. - Plenum Press, New York 1987.

Linkermer, G., Board, J.E., Musgrave, M.E.: Waterlogging effects on growth and yield components in late-planted soybean. - Crop Sci. **38**: 1576-1584, 1998.

Love, J.M., Knight, A.M., McAleer, M.A., Todd, J.A.: Toward construction of a high resolution map of the mouse genome using PCR-analyzed microsatellites. - Nucl. Acids Res. **18**: 4123-4130, 1990.

Makhmudov, S.H.A.: A study on chlorophyll formation in wheat leaves under moisture stress. - Field Crops Abstr. **39**: 1753, 1983.

Meksem, K., Leister, D., Peleman, J., Jabeau, M., Salamini, F., Gebhardt, C.: A high-resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. - Mol. Genet. Genomics **249**: 74-81, 1995.

Meksem, K., Ruben, E., Hyten, D., Triwitayakorm, K., Lightfoot, D.A.: Conversion of AFLP bands into high-throughtput DNA marker. - Mol. Genet. Genomics **265**: 207-214, 2001.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V., Aparicio-Tejo, P.: Drought induces oxidative stress in pea plants. - Planta **194**: 346-352, 1994.

Murakami, S., Matsui, K., Komatsuda, T., Furuta, Y.: AFLP-based STS markers closely linked to a fertility restoration locus (*Rfm1*) for cytoplasmic male sterility in barley. - Plant Breed. **124**: 133-136, 2005.

Oosterhuis, D.M., Scott, H.D., Hampton, R.E., Wullschleger, S.D.: Physiological responses of two soybean [*Glycine max* (L.) Merr.] cultivars to short-term flooding. - Environ. exp. Bot. **30**: 85-92, 1990.

Ortuño, M.F., Alarcón, J.J., Nicolás, E., Torrecillas, A.: Water status indicators of lemon trees in response to flooding and recovery. - Biol. Plant. **51**: 292-296, 2007.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A.: The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. - Mol. Breed. **2**: 225-238, 1996.

Reyna, N., Cornelious, B., Shannon, J.G., Sneller, C.H.: Evaluation of a QTL for waterlogging tolerance in southern soybean germplasm. - Crop Sci. **43**: 2077-2082, 2003.

Seo, Y.W., Jang, C.S., Johnson, J.W.: Development of AFLP and STS markers for identifying wheat-rye translocations possessing 2RL. - Euphytica **121**: 279-287, 2001.

Seong, R.C., Sohn, J.Y., Shim, S.I.: Responses of soybean cultivars to excessive soil moisture imposed at different growth stages. - Korean J. Crop Sci. **45**: 282-287, 2000.

Shan, X., Blake, T.K., Talbert, L.E.: Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat. - Theor. appl. Genet. **98**: 1072-1078, 1999.

Tautz, D.: Genomic fingerprinting goes simple. - BioEssays **12**: 44-46, 1990.

Thompson, J.E., Legge, R.L., Barber, R.F.: The role of free radicals in senescence and wounding. - New Phytol. **105**: 317-344, 1987.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M.: AFLP: a new technique for DNA fingerprinting. - Nucl. Acids Res. **23**: 4407-4414, 1995.

Wample, R.L., Davies, R.W.: Effect of flooding on starch accumulation in chloroplasts of sunflower (*Helianthus annuus* L.). - Plant Physiol. **73**: 195-198, 1983.

Yu, K.F. Pauls, K.P.: Optimization of DNA-extraction and PCR procedures for random amplified polymorphic DNA (RAPD) analysis in plants. - In: Griffin HG, Griffin AM (ed.): PCR Technology, Current Innovations. Pp. 193-200. CRC press, Boca Raton 1994.