Developing DNA Markers for Assisting Selection of Field Weathering Resistance in Soybean

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Abstract The deterioration of seed vigor as well as viability, due to high temperature and high relative humidity during the stages of the post-maturation and pre-harvest period is referred to as field weathering. Field weathering is the main limitation for producing high quality soybean seeds in the tropics and subtropics. The purpose of this study is to identify DNA markers linked to the field weathering resistant genes, and to develop markers for assisting selection in breeding program. The field weathering resistance of soybean variety Chiangmai 60 (susceptible), GC10981 (resistant) and 139 F_2 progenies derived from the cross of CM60/GC10981 was tested by modified incubator weathering and the controlled deterioration treatment. The seeds germination and viability of F_2 progenies showed normal distribution under both treatments. It hinted that the field weathering resistance was controlled by polygene. According to the seeds germination and viability of the F_2 progenies, six extremely resistant plants and seven extremely susceptible plants were pooled for bulk segregant analysis by AFLP markers. Five field weathering resistance linked polymorphism were identified from 2 162 AFLP markers. The 5 DNA fragments were cloned and sequenced. PCR primers were designed from the sequences to amplify the related DNA fragment from the genomic DNA of F_2 progenies. It was found that marker Eaag/Mcac-233 and Eact/Mctt-157 were in the same linkage group with a genetic distance of 25.8 cM. A major QTL controlling the field weathering resistance was identified between these two markers. The QTL located at 14 cM from marker Eaag/Mcac-233 and explained 29.7% of the variation in field weathering resistance. These two DNA markers have been used for assisting selection in breeding program as an attempt. Seven F_2 progenies were selected and backcrossed to CM60 using the developed markers in combination with field weathering resistance characters. The germination and viability of 18 BC_1F_1 progenies (41.9%) were higher than the mean of CM60 and GC10981 by controlled deterioration test. It is potentially possible to use these markers for assisting selection in breeding programs that focus on seed quality in the tropics.

Key words Soybean; Field weathering resistance; Bulked segregant analysis; Quantitative trait locus; Marker assisted selection

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与大豆田间老化抗性连锁的分子标记的发掘及辅助选择应用研究

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摘要 大豆种子成熟至收获期间如遇高温高湿天气,种子活力及活性会急剧下降,这就是所谓的田间老化(field weathering)。田间老化是热带、亚热带地区大豆生产的主要限制因素之一。本研究旨在寻找与田间老化性状相连锁的 DNA 标记并将其应用于辅助选择育种。为此,利用修改的培养箱老化法和人工控制老化法对大豆品种 Chiangmai 60 (敏感), GC10981 (抵抗) 及其 F_2 群体(139 个体)进行了鉴定。在两种处理条件下,F_2 代群体的种子发芽率及活性均为正态分布,说明大豆种子田间老化抗性受多个基因控制。根据 F_2 代个体的种子发芽率及活性,6 个高抗个体及 7 个高感个体的 DNA 分别被混合为抗性池和感性池,并利用 AFLP 标记进行了混合群体分析(Bulk Segregant Analysis)。从扩增的 2162 个标记中,发现了 5 个可能于大豆种子田间抗性相连锁的片段。通过 DNA 克隆和测序,设计了 5 对引物用于从大豆总 DNA 中扩增相应的片段。其中 3 对引物扩增的片段差异太小或未能扩增正确大小的片段,没能用于 F_2 群体。引物 Eaag/Mcac-233 和 Eact/Mctt-157 能扩增出差异明显的多态性,通过对 F_2 代群体的分析,这 2 个标记属同一连锁群,遗传距离为 25.8cM。QTL 分析结果显示有一个 QTL 位于这两个标记之间,距 Eaag/Mcac-233 约 14cM,可以解释 29.7% 的变异。用这两对引物对整个 F_2 群体进行筛选,20 个个体属于抗性群体,结合抗性鉴定的结果,7 个个体被用于与 Chiangmai 60 进行回交。18 个 BC_1F_1 个体(41.9%)的抗性高于其亲本的平均值。说明这些标记进行可以被用于大豆田间老化抗性的辅助筛选研究。

关键词 大豆;田间老化抗性;混合群体分析;数量性状位点;标记辅助选择

1 INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the world's leading sources of vegetable oil and plant protein. High temperature and moisture during post-maturation and pre-harvest period is a major obstacle to produce high quality soybean seeds in the tropics and subtropics[1]. Soybean seed vigor and viability reach a peak, and attains its highest potential quality at physiological maturity (maximum seed dry weight). Due to high moisture content (about 55%), unfortunately, the seed cannot be commercially harvested and must remain on the plant until a harvestable moisture level was reached[2]. This period may vary from a few days to over 3 weeks. Climatic conditions during this post-maturation and pre-harvest period have a great influence on the quality of the harvested seed[3].

Soybean seed quality deteriorates in the field prior to harvest in tropical regions, and the deterioration continues at a rapid rate after physiological maturity because of high temperature, high humidity and frequent or prolonged rainfall. Deterioration of seed vigor and viability due to high temperature and high relative humidity during the stages of seed physiological maturity and harvesting is referred to as field weathering[4]. Genotypic differences in resistance to field weathering have been observed. However, genetic differences appear to be small in comparison with the effect of environmental stress[2].

In recent years, significant progress has been made in soybean genomics to target important genes, which provides a deeper insight into its genome structure and organization. Based on the construction of soybean genetic maps, some quantitative trait loci (QTL) for seed quality in soybean have been mapped[5]. There are also some reports on environmental stress resistance of soybean, such as chilling tolerance[6]. DNA marker technology has been developed and integrated into soybean breeding programs, such as marker assisted selection (MAS). By using AFLP combined with bulk segregant analysis (BSA), Meksem et al.[7] identified molecular markers closely linked with the two major QTLs associated with soybean cyst nematode resistance. In soybean, some varieties have been found to be resistant to the field weathering[8]. Based on the prior researches, Chiangmai 60 (CM60) and GC10981 were chosen as susceptible and resistant variety for genetic analysis in this study. AFLP markers combining with BSA were used to identify the markers linking to the field weathering resistance. Furthermore, the obtained markers were used for MAS in a backcross breeding program as an attempt.

2 MATERIALS AND METHODS

2.1 Plant materials

Soybean Chiangmai 60 (CM60) and GC10981 were used as susceptible and resistant parents in this study. Kaowanant[8] had evaluated the field weathering resistance of these two varieties and indicated that CM60 was susceptible and GC10981 was resistant to field weathering.

2.2 Population development, sampling and field weathering test

Soybean CM60 and GC10981 were planted in a greenhouse as female and male parents for crossing. The obtained F_1 seeds were also grown in the greenhouse to produce F_2 seeds. The F_2 seeds were grown in a field at National Corn and Sorghum Research Center, Nakorn Rachasima Province, Thailand. Four weeks after planting, every F_2 plant was numbered and 1g leaf from each F_2 plant was collected for DNA extraction. At physiological maturity, the yellow pods were harvested from each F_2 plant for field weathering test. The field weathering resistance of each F_2 progeny was tested by incubator weathering and controlled deterioration methods as described by Dassou and Kueneman[9] and Ye et al[10].

2.3 DNA extraction and AFLP analysis

According to the field weathering resistance of the F_2 progenies, equal amounts of leaf tissue from extremely resistant plants were pooled as bulked resistant sample, while equal amounts of leaves from extremely susceptible plants were pooled as bulked susceptible sample in the same way. The genomic DNA of the parent, bulked samples and all F_2 plants were extracted by using the extraction protocol described by Keim et al[11]. AFLP analysis was conducted according to the procedures of Vos et al[12] and Maughan et al.[13]. The genomic DNA of the parent, bulked resistant and susceptible samples were digested with EcoRI and MseI, and amplified using E (ANN) and M (CNN) selective primers. The PCR products were separated in 6% denaturing polyacrylamide gel and stained by silver nitrate solution. The polymorphism was identified based on the visualization of the DNA bands. DNA bands showed linked polymorphism were excised and re-amplified. The PCR product was then cloned into the pGEM-T Easy Vector Systems (Promega Co.), transformed into *E. coli* XL-1 competent cell and sequenced following the manufacturer's protocols. The sequence of the cloned DNA fragment was used to design sequence characterized amplified region (SCAR) primers for checking the relationship between the markers and the field weathering resistance of all the F_2 progenies.

2.4 Marker assisted selection, backcrossing and efficiency evaluation

Based on the band pattern of the DNA amplified by SCAR primers, the F_2 progenies that showed the same DNA pattern as GC10981 were considered to be resistant to field weathering. The F_2 progenies showed resistant DNA pattern and resistant to field weathering (high germination and viability) were grown in a greenhouse as male parent and crossed to CM60. The ob-

tained BC_1F_1 seeds along with CM60, GC10981 and relative F_3 lines were grown in a field to compare the field weathering resistance of the BC_1F_1 plants and their parents. The physiological mature pods were harvested from each plant for field weathering test. The pods from each BC_1F_1 plant were treated separately, while the pods from each F_3 line were mixed as one sample. The pods were dried and threshed, and then 50 seeds of each BC_1F_1 plant and F_3 line were treated by controlled deterioration as described above. The efficiency of the marker assisted selection was evaluated by comparing the backcross progenies to CM60, GC10981 and corresponding F_3 lines.

2.5 Data analysis and QTL identification

The statistical analyses were carried out by using Microsoft Excel and SPSS 11.5 for Windows (SPSS Inc.). Genetic map distances were calculated using Mapmaker/EXP 3.0 computer program[14]. Information from Mapmaker was used in windows QTL Cartographer V2.5[15] to verify the candidate QTL by composite interval mapping (CIM). A location with a LOD score greater than 3.0 was considered to identify QTL significantly associated with the trait.

3 RESULTS

3.1 Field weathering resistance of F_2 progenies

The field weathering resistance of 139 F_2 progenies was evaluated by the germination and viability of the treated seeds using incubator weathering and controlled deterioration methods. For incubator weathering test, the germination of the F_2 plants ranged from 21.3 to 81.6%, whereas those of CM60 and GC10981 were 34.7% and 75%, respectively. The viability of the F_2 plants ranged from 47.8 to 95.6%, whereas those of CM60 and GC10981 were 59.7% and 93.4%, respectively. For controlled deterioration test, the germination of the F_2 plants ranged from 20 to 82%, whereas those of CM60 and GC10981 were 32% and 72%, respectively. The viability of the F_2 plants ranged from 44 to 90%, whereas those of CM60 and GC10981 were 54% and 94%, respectively. The germination and viability of both treatments showed normal distribution (absolute value of Skewness = 0.079 ~ 0.395, Kurtosis = 0.523 ~ 0.742). The field weathering resistance of soybean appeared to be a quantitative trait controlled by polygene. A significant correlation was observed between the incubator weathering and the controlled deterioration (germination $r = 0.331^{**}$, viability $r = 0.425^{**}$, $n = 139$, $P < 0.001$).

According to the germination and viability of the field weathering tests, six F_2 plants with extremely high germination and viability were bulked as resistant pool, while seven susceptible F_2 plants with extremely low germination and viability were bulked as susceptible pool for BSA.

3.2 Polymorphism detection and marker development

Totally, 2162 DNA fragments were amplified by using 64 E(ANN)-M(CNN) primer combinations, and 120 of them (5.6%) were polymorphic between CM60 and GC10981. Based on the bulk segregant analysis, five DNA fragments showed linked polymorphism with the field weathering resistance (Fig. 1). These 5 DNA fragments were cloned and sequenced. The sequence data were registered to the DNA database of Japan (DDBJ) (Table 1).

The SCAR primers from Eaag/Mcag-180 and Eagg/Mcac-115 showed a very small difference with a co-amplified band making it difficult to check the segregation in the F_2 progenies. The primers from Eagc/Mcag-104 could not amplify the

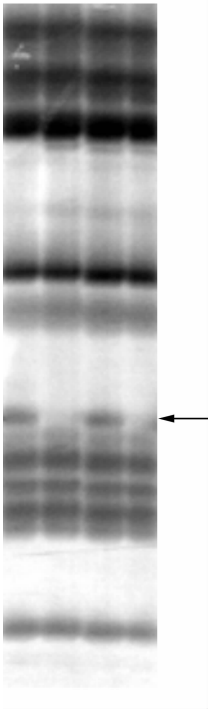

Fig. 1 An example of detecting linked polymorphism by bulk segregant analysis using AFLP primer combination Eagg/Mcac. Lanes from the left to right are GC10981, CM60, resistant bulk and susceptible bulk. The arrow indicates the linked polymorphism that GC10981 and resistant bulk showed DNA band, but CM60 and susceptible bulk showed no band.

Table 1 Clone information and SCAR primers of the sequenced DNA fragments

Clone name	Origin	DDBJ accession number	SCAR primer sequence
Eaag /Mcac-233	CM60	AB213662	Forward: 5′-ttaacaccaattgtcgtgcat-3′ Reverse: 5′-gaattcaaggacccttttact-3′
Eaag /Mcag-180	CM60	AB213663	Forward: 5′-gaattcaagctaacaagtttctct-3′ Reverse: 5′-ttaacagcagctgcaacaacaat-3′
Eact /Mctt-157	CM60	AB213664	Forward: 5′-gaattcactcagctgttacat-3′ Reverse: 5′-ttaacttgtccagcatgat-3′
Eagc /Mcag-104	GC10981	AB213665	Forward: 5′-ttaacaggaaaaggtacat-3′ Reverse: 5′-gaattcagccctttct-3′
Eagg /Mcac-115	GC10981	AB213666	Forward: 5′-gaattcagggtgttgaaat-3′ Reverse: 5′-ttaacaccataagaggttat-3′

correct fragment. The primers from Eaag/Mcac-233 and Eact/Mctt-157 showed a very clear polymorphic band with a co-amplified band. Therefore, these two markers were used to check the segregation in the F_2 progenies. For marker Eaag/Mcac-233, 108 F_2 progenies showed the same genotype as CM60, while the other 31 F_2 progenies showed the same genotype as GC10981 (fit for 3: 1, $\chi^2 = 0.540$, $p = 0.463$). For marker Eact/Mctt-157, 102 F_2 progenies showed the same genotype as CM60, while the other 37 F_2 progenies showed the same genotype as GC10981 (fit for 3 : 1, $\chi^2 = 0.194$, $p = 0.659$). The SCAR markers developed from AFLP marker were inherited in a Mendelian manner with dominant segregation patterns (3: 1). A t-test was carried out to compare the means of the germination and viability between the two genotypes, the P values for all tests were less than 0.001. There was a significant difference between the two genotypes. The germination and viability of the GC10981 genotype were higher than the CM60 genotype.

3.3 Genetic mapping and QTL identification

By linkage analysis, marker Eaag/Mcac-233 and Eact/Mctt-157 were in the same linkage group. The genetic distance between the markers was 25.8 cM. A QTL was identified by all the four indicators including IW germination, CD germination, IW viability and CD viability. The QTL explained 29% and 32% of the variation in the viability of the two weathering treatments. It was higher than those in the germination of both treatments (12% and 19%). This QTL may contribute more for viability than for germination. If the average score of germination and viability of both treatments was considered to be a composite index for field weathering resistance, a QTL could also be identifiedat 14 cM from marker Eaag/Mcac-233 with a LOD score of 9.4. This QTL explained 29.7% of the variation in weathering resistance (Table 2).

Table 2 The QTL position and genetic contribution for the germination and viability. The map distance was 25.8 cM beginning from marker Eaag/Mcac-233 (0 cM) to marker Eact/Mctt-157 (25.8 cM), and the QTL position was the peak LOD score from marker Eaag/Mcac-233

Weathering indicator*	QTL position /cM	LOD score	Additive effect	R^2/%
IW germination	18.0	3.4	-5.518	11.9
CD germination	10.0	5.1	-7.850	18.7
IW viability	16.0	9.0	-6.002	29.2
CD viability	12.0	9.0	-6.270	31.8
Overall resistance	14.0	9.4	-6.107	29.7

* IW = incubator weathering; CD = controlled deterioration; Overall resistance is the mean of the germination and viability of both treatments.

3.4 Marker Assisted Selection, Backcrossing and efficiency evaluating

According to the DNA band pattern of SCAR marker Eaag/Mcac-233 and Eact/Mctt-157, among the 139 tested F_2 progenies, 91 progenies presented bands by both markers (A_B_), 28 progenies presented bands by only one marker (A_bb or aaB_), and 20 progenies showed no band by either marker (aabb). By one-way ANOVA test, all the P values were less than 0.05, most of the P values were less than 0.001. There was a significant difference among these three genotypes. In both treatments, the aabb genotype (same as GC10981) had a higher germination and viability than other heterozygotes and homozygotes.

By comparing the germination and viability of the 20 F_2 progenies that showed same DNA pattern as GC10981 (absent band at both markers), seven F_2 progenies with high germination and viability were selected for backcrossing to the recurrent parent CM60. Totally 43 BC_1F_1 plants were developed. The yellow pods from the 43 BC_1F_1 plants were harvested separately for field weathering evaluation by controlled deterioration test. The germination of BC_1F_1 plants ranged from 28 to 78%, while those of CM60 and GC10981 were 30% and 74%, respectively. The viability of the BC_1F_1 plants ranged from 58 to 94%, while those of CM60 and GC10981 were 56% and 90%, respectively.

Table 3 A comparison of the germination and viability of the F_3 line and the BC_1F_1 family derived from the same F_2 plant. All the seeds were treated by controlled deterioration method

Line name	F_2		F_3		BC_1F_1 (Average)	
	Germination/%	Viability/%	Germination/%	Viability/%	Germination/%	Viability/%
A10	82	90	56	78	49.3	77.7
A11	74	86	66	88	56.8	85.2
A35	76	90	68	82	50.0	78.5
A63	72	90	70	88	53.1	77.8
C2	76	90	58	84	45.3	73.3
G20	70	82	72	92	50.3	77.1
G21	62	86	62	86	50.0	74.9
CM60	32	54	26	58	30.0	56.0
GC10981	72	94	76	96	74.0	90.0

The average germination and viability of the BC_1F_1 plants derived from the same F_2 plant (F_3/CM60) were calculated and compared with the germination and viability of the relative F_2 progenies and F_3 lines (Table 3). By paired t-test, the correlation between the different generations was not significant ($p > 0.05$). This might be due to the limited sample number. The difference between F_2 progenies and F_3 lines was not significant ($t_6 = 2.228$, $p = 0.067$ for germination and $t_6 = 0.834$, $p = 0.436$ for viability), but the difference between F_2 progenies and BC_1F_1 families was signicant ($t_6 = 7.862$, $p < 0.001$ for germination and $t_6 = 4.948$, $p = 0.003$ for viability). There was also a significant difference between F_3 progenies and BC_1F_1 families ($t_6 = 6.992$, $p < 0.001$ for germination and $t_6 = 3.744$, $p = 0.010$ for viability). The germination and viability of the treated seeds of BC_1F_1 were lower than those of the F_2 progenies and F_3 lines. By comparing the germination and viability of different generations, the field weathering resistance of F_3 lines was weaker than the relative F_2 progenies due to the genotypic segregation. After the F_3 plants were backcrossed to CM60, the field weathering resistance of BC_1F_1 families (mean) further decreased due to the permeation of the CM60 background. However, 41 of the 43 the BC_1F_1 progenies still showed higher germination and viability than CM60. The germination and viability of 18 BC_1F_1 progenies (41.9%) were higher than the mean of CM60 and GC10981 by controlled deterioration test.

4 DISCUSSION

The use of BSA in combination with AFLP method has been proved to be a very useful and powerful technique for identifying markers that are tightly linked to, or cosegregated with, genes underlying monogenic traits[16]. The limitation is the difficulty of using AFLP markers on large population directly. Meksem et al.[17] had successfully converted AFLP band into STS which provides an efficient tool for genomic mapping and marker assisted breeding. But the loss of the original polymorphism during generation of the STS and loss of the locus specificity of the STS is still an experimental challenge in generating sequence-specific STSs from AFLP bands. In this study, though we successfully identified 5 polymorphic AFLP markers linked to the field weathering resistance of soybean, only two of the five sequenced fragments were successfully transfer into SCAR markers. The segregation and linkage analysis indicated that the SCAR markers developed from AFLP markers were inherited in a Mendelian manner with dominant segregation patterns (3∶1). The dominant homozygotes (CM60 genotype) and heterozygotes could not be distinguished by these dominant markers. However, since the recessive homozygotes (GC10981 genotype) were considered to be resistant to field weathering, it is possible to select the resistant homozygotes by these markers in breeding programs.

Michelmore et al.[16] suggested that BSA can be used for "genomic walking" to develop markers for genetic mapping. In order to look for more markers linking to the two markers developed from this study and to fine mapping QTLs controlling the field weathering resistance of soybean, we registered our sequence data to DNA database of Japan (DDBJ) and compared them with the soybean sequence data from DDJB by BLAST and FASTA analysis. By BLAST searching, only accession BU765372 and BU549864 were found to have 102bp same as marker Eact/Mctt-157 within 118bp overlap (86.4%). By FASTA searching, accession AF180335 and AF186186 were found to have discontinuous similarity (80bp and 129bp) with marker Eaag/Mcac-233, accession AF243378 has partly discontinuous similarity (46bp) with marker Eact/Mctt-157. No sequence was found similar to marker Eaag/Mcag-180, Eagc/Mcag-104 and Eagg/Mcac-115 by both BLAST and FASTA. It was clear that these sequences were not the same as the existed sequences in the database. All the similar accessions were cDNA clones from soybeans, but there was no chromosomal information about these clones. Thus, the chromosomal positions of the markers developed in this study could not be identified by current searching from the database.

Although it is possible to use either marker or agronomic character for assisting selection in breeding programs[18], especially at an earlier stage, however, a selection index that includes both phenotypic measurement and a molecular marker score can increase the selection response relative to phenotypic selection alone, particularly if much of the additive genetic variance in a character can be explained using the molecular markers. In this study, when only the markers were used for selection, 20 F_2 progenies were selected. After combining the DNA marker selection with the field weathering resistance (germination and viability) for selection, only seven progenies that highly resistant to the field weathering were selected for backcrossing. Most of the backcrossed progenies were still more resistant to field weathering resistance than CM60. Thus, this selection seems to be efficient. However, more markers and larger population is needed to make an efficient selection in the later generations.

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