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OPTIMIZATION OF DOT-BLOT SNP ANALYSIS FOR THE DETECTION OF DROUGHT OR SALINITY STRESS ASSOCIATED MARKER IN FOXTAIL MILLET (*Setaria italica* L.)

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SUMMARY

Foxtail millet (*Setaria italica* L.) is an underutilized crop grown for its nutritious grains and relative tolerance to drought or salinity stresses. However, variation in the tolerance level exists among foxtail millet genotypes. A simple and reliable technique for SNP genotyping, namely dot-blot SNP analysis, has been applied for practical plant breeding programs and has a potential for accelerating foxtail millet breeding for drought or salinity stress tolerance. The aim of this study was to conduct SNP analysis for the *SiDREB2* gene, which is associated with drought or salinity stress tolerance in foxtail millet, using a marker based on polymorphism at the 558th nucleotide,. Two factors that affect the allele-specific detection of the dot-blot SNP analysis i.e. hybridization temperature and competitive probe ratio were optimized in this study. Four hybridization conditions consisting of the combination between two hybridization temperatures (50 and 55 ºC) and two competitive probe ratios (1:5 and 1:10) were optimized. The second hybridization condition (50 ºC hybridization temperature and 1:10 competitive probe ratio) showed the best result for SNP analysis. This optimum condition was then applied for genotyping 26 foxtail millet genotypes with unknown drought or salinity stress tolerance levels. The optimum condition of the dot-blot SNP analysis was effective for genotyping in an allele-specific manner and used for predicting stress tolerance levels of the foxtail millet genotypes. The results of this study are useful for accelerating foxtail millet breeding for drought or salinity stresses in the future.

Key words: Optimization, hybridization conditions, SNP analysis, drought or salinity stress

Key findings: A dot-blot based SNP analysis method was developed for the prediction of drought or salinity stress tolerance in foxtail millet genotypes and was applied to 26 genotypes. This information will be useful for foxtail millet breeding.

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INTRODUCTION

Foxtail millet (*Setaria italica* L.) is a small-grained cereal which has short life duration, and needs low inputs, remaining free from pest and disease (Upadhyaya *et al.,* 2008). Grains produced by this plant have protein content two times higher than that of rice, rich in dietary fiber and minerals e.g. copper and iron for boosting immunity (Dayakar-Rao *et al.,* 2016). There are studies reported the health benefits of foxtail millet grains, including stabilizing blood sugar content, reducing blood cholesterol levels, and anti-carcinogenic effects in human colon cancer cells (Sireesha *et al.,* 2011; Shan *et al.,* 2015). Foxtail millet is reported to be more tolerant to drought and salinity stresses than the other cereal plants i.e. barley, maize, rice, and wheat (Ayers *et al.,* 1952; Panaud 2006; Islam *et al.,* 2011). Thus, foxtail millet could potentially provide a source of alternative and functional foods that can be grown in marginal lands exposed to drought and salinity stress.

Drought and salinity are two major abiotic stresses affecting crop production worldwide. It is estimated that 40% land areas are affected by drought, while the other 20% were affected by salinity stress, respectively (Zhang *et al.,* 2014; Shrivastava and Kumar 2015). Both of these stresses alter the physiological, biochemical, and molecular activity of plants and affect plant growth and productivity (Wang *et al.,* 2003). Several genes have been extensively studied to reveal the stress tolerance mechanisms of plants. Transcription Factors (TF) are a group of proteins that act as regulators to the expression of functional genes (i.e. water and ion transport, cellular membrane integrity, and macromolecule protection) which are directly involved in plant stress tolerance mechanisms (Nakashima *et al.,* 2012; Li *et al.,* 2016).

Dehydration Responsive Element Binding 2 (*DREB2*) is one of the TF genes whose expression is induced by dehydration or high salt stress (Sakuma *et al.,* 2006). A homolog of the *DREB2* gene has been identified and characterized in foxtail millet as *SiDREB2.* There are differences between the expression of the *SiDREB2* gene in tolerant and sensitive foxtail millet cultivars, suggesting a role for this gene in stress tolerance mechanisms (Lata *et al.,* 2011). Association studies have revealed that there is A/G transition at the 558th nucleotide of the *SiDREB2* gene that is able to distinguish foxtail millet cultivars based on their drought or salinity stress tolerance levels. A study conducted by Putri (2017) on four foxtail millet genotypes revealed that tolerant genotypes namely I-5 and I-6 have an A allele, and sensitive genotypes namely I-4 and I-10 have a G allele. This SNP marker has been

reported to be linked with a lipid peroxidase content trait, which serves as a biochemical marker for dehydration stress tolerance in foxtail millet (Lata and Prasad, 2013).

SNPs that associate with phenotypes could potentially be developed into molecular markers to assist plant breeding programs. Most of SNP genotyping techniques require expensive and specialized instruments which are not available in most molecular biology laboratories (Liu *et al.,* 2012). However, dot-blot SNP analysis was considered as highly reliable, simple, and cost-efficient SNP analysis technique and does not require elaborate equipment. This technique has been applied for genotyping individuals in practical breeding programs in Japan (Shirasawa *et al.,* 2006). Before analyzing the SNP, optimizations were performed to enable allele-specific detection of the dot-blot SNP analysis. Two factors that affect the allele-specific detection by the dot-blot SNP analysis i.e. hybridization temperatures and competitive probe ratios (Matsubara and Kure 2003; Shirasawa *et al.,* 2006), were optimized in this study.

In this paper we report the first application of the dot-blot SNP technique for SNP analysis in foxtail millet. Optimum hybridization condition, consisting of an appropriate hybridization temperature and competitive probe ratio was applied for genotyping SNP marker associated with drought or salinity stress tolerance. The aim of this study was to predict, based on the results of SNP analysis, the drought or salinity stress tolerance levels of 26 foxtail millet genotypes for which there is no relevant published information. This information will support the acceleration of breeding programs for drought or salinity stress tolerance in foxtail millet.

MATERIALS AND METHODS

Genetic materials

Four foxtail millet genotypes for which the drought or salinity stress tolerance levels and SNP genotypes have been revealed according to Putri (2017) were used as references. Those are: I-5 and I-6 (tolerant, A allele); I-4 and I-10 (sensitive, G allele). Twenty-six foxtail millet genotypes from Indonesia of unknown abiotic stress tolerance levels were also analyzed using optimum condition obtained in this study (Table 1).

Germination assay of four foxtail millet genotypes at seedling stage under drought or salinity stress

In this experiment the seeds of the foxtail millet genotypes (i.e. I-4, I-5, I-6, and I-10) were germinated in the drought or salinity stress condition. The drought condition was mimicked by adding 144.5 g of PEG 6000 (Polyethylene Glycol, Mol. Wt. 6000) to 1000 mL of distilled water to reduce the water potential up to -5.0 MPa according to Hadas (1976). The saline condition was made according to Ardie *et al.* (2015) by using 100 mM NaCl. The experiment was arranged with Completely Randomized Design (CRD) with single factor i.e. foxtail millet genotypes (I-4, I-5, I-6, and I-10) and replicated three times. Ten seeds of each foxtail millet genotype were sown in the petridish covered by filter paper according to Bayoumi *et al.* (2008). Stress conditions i.e. drought or salinity were applied since the first

day of experiment. The length of the root and shoot were measured in 7 Days After Treatment (DAT). The data were analyzed using Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) and T-Test at 0.05 significance levels. The data were analyzed using R Statistics software version 3.3.1.

Table 1. List of the foxtail millet genotypes used in this study.

Genotypes ID	Name	Origin
1	ICERI-1	Indonesian Cereal Research Institute
2	ICERI-2	Indonesian Cereal Research Institute
3	ICERI-3	Indonesian Cereal Research Institute
4	ICERI-7	Indonesian Cereal Research Institute
5	ICERI-8	Indonesian Cereal Research Institute
6	ICERI-9	Indonesian Cereal Research Institute
7	Botok-2	East Nusa Tenggara, Indonesia
8	Botok-4	East Nusa Tenggara, Indonesia
9	Botok-5	East Nusa Tenggara, Indonesia
10	Botok-6	East Nusa Tenggara, Indonesia
11	Botok-7	East Nusa Tenggara, Indonesia
12	Botok-10	East Nusa Tenggara, Indonesia
13	Botok-11	East Nusa Tenggara, Indonesia
14	Botok-12	East Nusa Tenggara, Indonesia
15	Botok-13	East Nusa Tenggara, Indonesia
16	Botok-14	East Nusa Tenggara, Indonesia
17	Botok-15	East Nusa Tenggara, Indonesia
18	Botok-16	East Nusa Tenggara, Indonesia
19	Botok-17	East Nusa Tenggara, Indonesia
20	Botok-18	East Nusa Tenggara, Indonesia
21	Botok-19	East Nusa Tenggara, Indonesia
22	Belitung	Belitung Island, Indonesia
23	Belitung-hitam	Belitung Island, Indonesia
24	Padang	West Sumatra, Indonesia
25	Ruilak	East Nusa Tenggara, Indonesia
26	T. Labapu	East Nusa Tenggara, Indonesia

Table 2. List of the primers and the probes used in this experiment.

Types	Sequences name	Nucleotide sequences
SiDREB2 gene primer	SiDREB2-DB-Fw	5'-TGCTGCATTGCACGGGTTTAATG-3'
	SiDREB2-DB-Rv	5'-GTCATAACTTACGTTCCCTTCT-3'
Dot-blot SNP analysis probe	Probe A	5'-GTACTTCA**A**TCTGAGGA*TATATT***AC GAGAAGGATGAAGCTTCTTTTCA**-3'
	Probe G	5'-GTACTTCA**G**TCTGAGGA*TATATT***TAC ATTCGCAATTAAGAGGCTTCGT**-3'

(a) Sequences printed in underline indicates the *SiDREB2* gene-specific sequence, nucleotide printed in bold within indicates the target SNP; (b) sequences printed in *italic* indicates the spacer sequence: (c) sequences printed in bold and underlined indicates the bridge sequence.

Probe design for the Dot-blot SNP analysis

The probes used in this experiment were designed using the *SiDREB2* gene sequences from four foxtail millet genotypes namely I-5 (GenBank ID: KY404097), I-6 (GenBank ID: KY404097), I-4 (GenBank ID: KY404096), and I-10 (GenBank ID: KY404101). The gene sequences were aligned using Geneious, version 10.0.3 (Biomatters Ltd., New Zealand). Probes for the dot-blot SNP analysis were designed according to Shiokai *et al.* (2010). Each probe consists of 48 nucleotides (17-nt gene specific sequence, 6-nt spacer sequence, and 25-nt bridge sequence). Two probes, namely A and G probes were designed to detect A or G alleles, respectively (Table 2).

Extraction of genomic DNA and PCR amplification

The Genomic DNA samples, used in this research, were extracted using CTAB method (Murray and Thompson 1980) and were diluted in TE buffer to 10 ng/µL concentration. The amplification of the DNA fragments was performed in 20 µL final volume of PCR reaction by using the TP600 Thermal Cycler (TaKaRa, Japan). The final reaction mixture consisted of 1 µL of genomic DNA (10 ng/ µL), 1 µL of forward and reverse primer (10 pmol each), 2 µL of 10× PCR buffer, 1.6 µL of 25 mM dNTP mix, and 0.1 µL homemade *Taq* polymerase. Two primers, namely SiDREB2-DB-Fw and SiDREB2-DB-Rv were used to amplify the *SiDREB2* gene fragment that contains a SNP (Table 2). The PCR profile was as follows: initial denaturation for 2 minutes at 94 ºC, 35 cycles of denaturation for 30 seconds at 94 ºC, annealing for 1 minute at 50 ºC, and extension for 30 seconds at 72 ºC. After the end of the cycles, final extension was performed for 7 minutes at 72 ºC.

Dot-blot SNP analysis

The PCR products of the *SiDREB2* gene fragment were mixed with alkaline solution (0.4 N NaOH and 10 mM EDTA) in 1:1 ratio. The mixtures of PCR product and alkaline solution were bloted into the nylon membranes (Pall Laboratory, USA) by using the Multi-Pin Blotter (ATTO Corporation, Japan) and then replicated twice. The membranes with blotted DNA fixed by using the GS Gene Linker UV Chamber (Bio-Rad, USA) and pre-hybridized with 10 mL hybridization buffer consisting of 20× saline sodium citrate (SSC), 0.1% SDS, 10% sarcosyl, and 1 g blocking reagent (Roche, Switzerland). The pre-hybridization was carried out on 50 ºC or 55 ºC for 1 hour, followed by an overnight hybridization in buffer containing 1 µL of the target probe and 5 or 10 µL of the competitive probe to make the final ratio of 1:5 or 1:10, and 1 µL of a digoxygenin (DIG) labeled probes. For example, to make a hybridization buffer to detect sample with the A allele, 1 µL of the A probe mixed with 5 or 10 µL of the G probe. Hybridizations were carried out on HB-80 Hybridization Incubator (Taitec Corporation, Japan). After an overnight hybridization, membranes were washed with the washing buffer (0.1× SSC and 0.1% SDS) under the same temperatures as the previous hybridization temperatures. Finally, signals were detected by using the DIG Nucleic Acid Detection Kit (Roche, Switzerland) and then captured using X-Ray films (Fujifilms, Japan).

RESULTS

Germination assay of four foxtail millet genotypes under drought or salinity stresses

The germination assay was performed to confirm the stress tolerance levels of four foxtail millet genotypes used as references. Based on the previous studies, I-5 and I-6 were identified as the tolerant genotypes with A allele, the I-4 and I-6 were identified as sensitive genotypes with G allele (Figure 1). Four foxtail millet genotypes showed significant differences in the root and shoot length, either in drought or salinity stresses (Table 3). The foxtail millet genotypes were separated into two groups, based on their tolerance to the drought or salinity stress i.e. tolerant (I-5 and I-6) and sensitive group (I-4 and I-10) for the combined

Table 3. Root and shoot length of four foxtail millet genotypes under drought or salinity stresses at 7 days after planting.

Genotypes	Drought stress		Salinity stress	
	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
I-5	3.95[a]	2.95[a]	1.45[a]	1.34[a]
I-6	4.16[a]	3.00[a]	1.35[ab]	1.3[a]
I-4	3.17[b]	1.48[c]	1.16[b]	0.92[b]
I-10	0.97[c]	2.25[b]	0.65[c]	0.51[c]

Means followed by the same letter within each column are not significantly different based on DMRT ($P < 0.05$)

Table 4. Root and shoot growth based on the combined analysis of the tolerant genotypes group (I-5 and I-6) and sensitive genotypes group (I-4 and I-10) under drought or salinity stresses.

Tolerance group	Drought stress		Salinity stress	
	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
Tolerant group	4.05[a]	2.97[a]	1.39[a]	1.32[a]
Sensitive group	2.24[b]	1.89[b]	0.91[b]	0.71[b]

Means followed by the same letter within each column are not significantly different based on T-Test ($P < 0.05$)

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                    541       550       560       570       580       590       600
                     |         |         |         |         |         |         |
I-5 (Tolerant)      TCCGTGGAGGTACTTCAATCTGAGGAGATTGTTTTACAGAAAGAAGGGAACGTAAGTTAT
I-6 (Tolerant)      TCCGTGGAGGTACTTCAATCTGAGGAGATTGTTTTACAGAAAGAAGGGAACGTAAGTTAT
I-4 (Sensitive)     TCCGTGGAGGTACTTCAGTCTGAGGAGATTGTTTTACAGAAAGAAGGGAACGTAAGTTAT
I-10(Sensitive)     TCCGTGGAGGTACTTCAGTCTGAGGAGATTGTTTTACAGAAAGAAGGGAACGTAAGTTAT
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Figure 1. The alignment of the part of the *SiDREB2* gene sequences from four foxtail millet genotypes with known drought or salinity stress tolerance levels (tolerant or sensitive). The underlined sequence indicates 17 nucleotides used for probes design. The nucleotides typed in bold indicate the SNP associated with stress tolerance levels of foxtail mllet.

analysis. The result of the combined analysis in both drought and salinity stresses showed significant differences between the two groups for the root and shoot length (Table 4). The germination assay confirmed the stress tolerance levels of four foxtail millet genotypes (Figure 2), as well as the association of a SNP in the 558th nucleotide of the *SiDREB2* with drought or salinity stress tolerance in foxtail millet.

Optimization of the Dot-blot SNP analysis

The A/G SNP at the 558th nucleotide of the *SiDREB2* in the four foxtail millet genotypes was associated with their levels of tolerance to drought or salinity stresses (Figure 1). Two probes namely A and G probes were designed for the SNP analysis at that marker. The A probe was designed to detect genotypes with an A allele, the G probe was designed to detect genotypes with the G allele. Four hybridization conditions consisting of the combination between two hybridization temperatures (50 and 55 ºC) and two competitive probe ratios (1:5 and 1:10) were optimized in this study (Table 5). The hybridization temperatures and competitive probe ratios tested for the optimization in this study were adapted from Shirasawa *et al.* (2006) and Shiokai *et al.* (2010). Those of the two hybridization temperatures showed the best result for SNP analysis compared to either the lower or higher temperatures in the previous studies, while the 1:5 or 1:10 competitive probe ratios were used as a standard. Four foxtail millet genotypes, in which their *SiDREB2* gene sequence was used for designing probes and had known stress tolerance levels were used as a reference for the optimizations of the SNP analysis. The results of the dot-blot SNP analysis optimization are shown in Figure 3.

Four hybridization conditions showed different results for SNP analysis. Under three of the four hybridization conditions, non-specific or weak signals were obtained. The first and the third conditions resulted in non-specific signals, while the fourth condition gave weak signals. Weak or non-specific signals could lead into false detection of allele and will not be used for dot-blot SNP analysis. However, under the second condition (50 ºC and 1:10 competitive probe ratio), clear and strong allele-specific signals were obtained (Figure 3). The genotypes with the A allele only detected by the A probe and the genotypes with the G allele only detected by the G probe. Based on the optimization of the hybridization conditions, the second condition gave the best result for analyzing SNP marker at the 558th nucleotides of the *SiDREB2* gene. This condition was used for genotyping the 26 foxtail millet genotypes with unknown drought or salinity stress tolerance levels.

Genotyping foxtail millet genotypes using the optimum condition and prediction of the drought or salinity stress tolerance levels

The second hybridization condition was used for examining the SNP in the *SiDREB2* gene of the 26 foxtail millet genotypes. The results of genotyping using the second condition are shown in Figure 4. The A probe was designed for genotyping plants with the A allele, the G probe was designed for genotyping plants with the G allele.

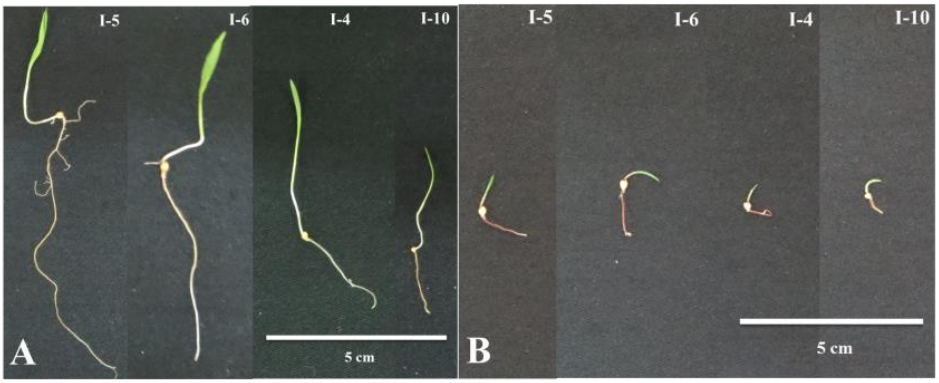

Figure 2. Root and shoot growth differences between four foxtail millet genotypes (I-5, I-6, I-5, and I-10) under drought (A) or salinity stress (B).

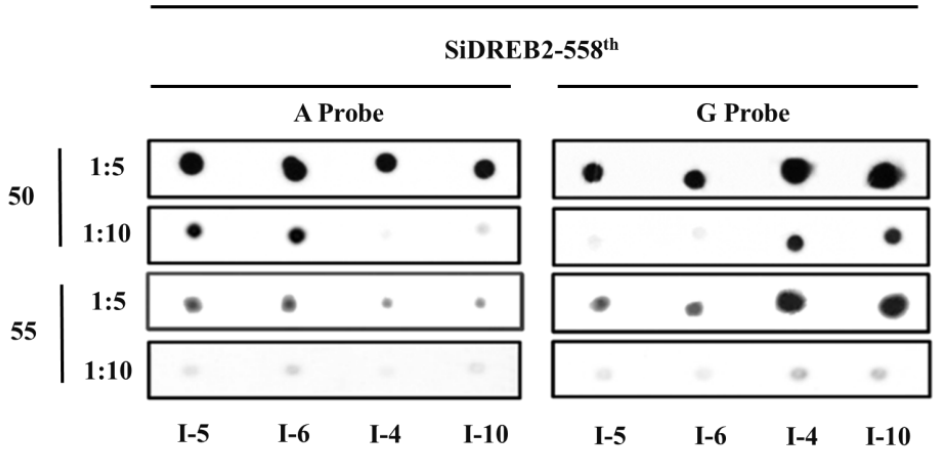

Figure 3. The results of the optimizations of the dot-blot SNP analysis for genotyping SNP marker at the 558th nucleotide of the *SiDREB2* gene using four cultivars, drought or salinity stress tolerance of which is already known. The A probe was designed for detecting tolerant genotypes with an A allele (I-5 and I-6). The G probe was designed for detecting sensitive genotypes with the G allele (I-4 and I-10). The numbers on the left side of the picture indicate the hybridization temperatures (50 and 55 ºC) and the competitive probe ratios (1:5 and 1:10).

Table 5. Four hybridization conditions used in this experiment

Conditions	Temperature (˚C)	Competitive probe ratios
I	50	1:5
II	50	1:10
III	55	1:5
IV	55	1:10

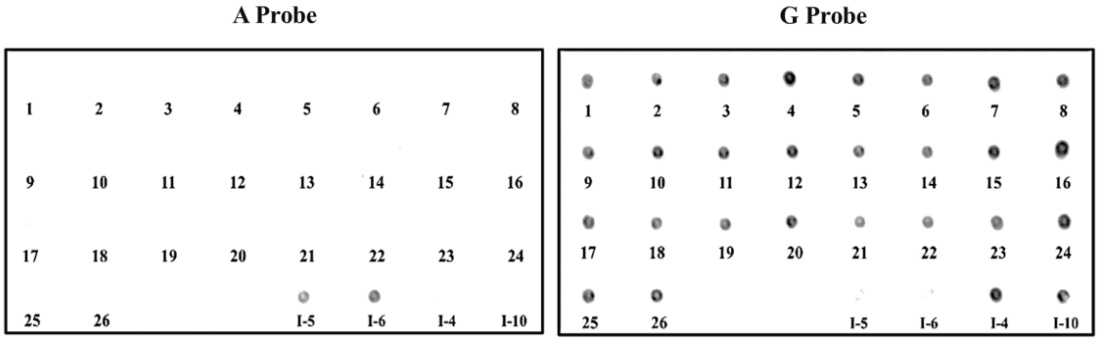

Figure 4. The results of the dot-blot SNP analysis using the SNP marker at the 558th nucleotide of the *SiDREB2* gene using twenty-six foxtail millet genotypes with unknown stress tolerance level. I-5 and I-6 were used as positive controls for the A probe, while I-4 and I-10 were used as negative controls. I-4 and I-10 were used as positive controls for the G probe, while I-5 and I-6 were used as negative controls.

Table. 6. The results of the SNP marker genotyping by the dot-bot SNP analysis and stress tolerance levels prediction of the 26 foxtail millet genotypes.

Genotypes ID	Names	Genotype	Predicted stress tolerance levels
1	ICERI-1	G	Sensitive
2	ICERI-2	G	Sensitive
3	ICERI-3	G	Sensitive
4	ICERI-7	G	Sensitive
5	ICERI-8	G	Sensitive
6	ICERI-9	G	Sensitive
7	Botok-2	G	Sensitive
8	Botok-4	G	Sensitive
9	Botok-5	G	Sensitive
10	Botok-6	G	Sensitive
11	Botok-7	G	Sensitive
12	Botok-10	G	Sensitive
13	Botok-11	G	Sensitive
14	Botok-12	G	Sensitive
15	Botok-13	G	Sensitive
16	Botok-14	G	Sensitive
17	Botok-15	G	Sensitive
18	Botok-16	G	Sensitive
19	Botok-17	G	Sensitive
20	Botok-18	G	Sensitive
21	Botok-19	G	Sensitive
22	Belitung	G	Sensitive
23	Belitung-hitam	G	Sensitive
24	Padang	G	Sensitive
25	Ruilak	G	Sensitive
26	T. Labapu	G	Sensitive

The genotypes with known allele were used as the positive controls for genotyping. The results of genotyping with the A probe showed that there were no foxtail millet genotypes that have an A allele. When the G probe was used, positive signals were present in all the samples. All of the genotypes used in this research were detected as either homozygous A or G by the dot-blot SNP analysis. This might have happened as the seeds of the plants used in this study were propagated by self-pollination. However, it is also possible to analyze heterozygous individuals by the dot-blot SNP analysis, since SNP markers are inherited as co-dominant (Xu 2010). Shirasawa *et al.* (2006) and Suzuki *et al.* (2011) demonstrated the utilization of the dot-blot SNP analysis for examining heterozygous individuals in the segregating population of rice. The results of the analysis of the SNP analysis in the 26 genotypes and their predicted tolerance to drought or salinity stresses are summarized in Table 6.

DISCUSSION

Selection for abiotic stresses tolerance using conventional methods is considered to be more complicated and difficult than selection for the other traits. This is caused by the dependency of stress tolerance mechanisms to the growth stage of the plant (Fita *et al.,* 2015). Screening for drought or salinity stress tolerance is also known to be laborious and time consuming (Mantri *et al.,* 2014). Simple selection methods, which are not dependent on the growth stage of the plant, not affected by environment, and less laborious should be utilized. The DNA markers could fulfill these requirements. Since the DNA of the plants can be obtained since the seedling stage, genotyping can be conducted in early growth stage of the plants and can be utilized for the elimination of the plants with undesired genotypes. DNA markers will enable us to simplify the phenotype screening and save cost, time, resources, and effort (Collard and Mackill 2008). The dot-blot SNP analysis is simple, rapid, and useful to genotype foxtail millet genotypes.

There are the other simple SNP genotyping methods that have been applied in plant improvement programs i.e. Allele-Specific PCR (AS-PCR) (Drenkard *et al.,* 2000) and Cleaved Amplified Polymorphic Sequence (CAPS) (Konieczny and Ausubel, 1993). The AS-PCR was the simplest technique for SNP analysis compared to the others, which only need a standard PCR using mismatched primers and followed by gel electrophoresis (Kim *et al.,* 2005). However, the AS-PCR technique requires specific properties of the DNA polymerase (Waters *et al.,* 2008; Liu *et al.,* 2012) and the reliability of the data obtained by this technique was questionable, because the results depend on the success of the DNA amplification by PCR (Tonosaki *et al.,* 2013). The CAPS technique requires at least three steps for SNP analysis i.e. DNA amplification by specific primers, digestion of amplified DNA using suitable restriction enzymes, and finally fragments separation using electrophoresis (Shavrukov, 2016). The main limitation of this technique is its dependency on the availability of the restriction sites present at the site of SNP (Kim *et al.,* 2005). When the levels of polymorphism are too low, the cost of the CAPS analysis increased due to the limitation of

restriction enzyme choices. Sometimes rare type of the restriction enzymes that recognize the specific restriction sites come with high price (Shavrukov, 2015; Shavrukov, 2016). Although the steps of the dot-blot SNP analysis are more laborious than the other two techniques, the data generated by this technique is highly reliable and an unlimited number of the SNP markers can be analyzed. The most important advantage of the dot-blot SNP analysis is that more number of the samples can be analyzed at one time, compared to the other two techniques. The dot-blot SNP analysis was able to analyze 864 samples on the membrane with 8 × 12 cm^2 size (Shirasawa *et al.,* 2006).

The results of this study may eliminate the phenotype screening of stress tolerance levels of the 26 foxtail millet genotypes, which is the most important but laborious step in plant breeding programs and can be extended to other crop species. This will help to accelerate the progress of the foxtail millet breeding programs for the drought or salinity stress. The purpose of our foxtail millet breeding is to develop cultivars that are tolerant to drought or salinity stress and have high grain qualities. The availability of DNA markers, associated with various agronomically important traits, will be useful for DNA-selection based breeding method (Shirasawa *et al.,* 2006), which will enable us to select plants based on the results of the DNA analysis without observing plant performances on the fields. Thus, the cost and time spent on the breeding programs will be reduced.

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