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Development of AFLP and STS Markers Related to Stay Green Trait in Multi-Tillered Maize

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ABSTRACT: In order to develop molecular markers related to stay green phenotype, AFLP analysis was conducted using near-isogenic lines for either stay green or non stay green trait. Both lines have characteristics of multi-ear and tillers (MET). Two out of 64 primer combinations of selective amplification identified three reproducible polymorphic fragments in MET corn with stay green. Both of E+AGC/M+CAC and E+AAG/M+CAA primer combinations produced two and one specific polymorphic fragments linked to stay green trait, respectively. For the conversion of AFLPs to sequence tag sites (STSs), primers were designed form both end sequences of each two polymorphic fragments. One fragment, which was amplified with E+AAG/M+CAA primer combinations, possessed 298 bp long and showed a 91% homology with maize retrotransposon *Cinful-1*. One out of two polymorphic fragments produced with E+AGC/M+CAC primer combination had 236 bp long and matched a 96% homology with an intron region of 22 kDa alpha zein gene cluster in *Zea mays*. One out of two PCR fragments amplified with MET2 primer set in the stay green MET was not produced in the non-stay green MET. The developed AFLP and STS marker could be used as an efficient tool for selection of the stay green trait in the MET inbred.

Keywords: AFLP, MET maize, stay green trait, STS

Leaf senescence is an important trait during development and growth stage Photosynthetic capacity of plants was progressively declined by subsequent leaf yellowing which was caused by the degradation of chlorophyll and chloroplast through programmed cell death Several researchers have reported stay green mutants that maintain leaf greenness during the grain ripening stage in both monocot and dicot such as maize (Gentinetta *et al.*, 1986), *Festuca pratensis* (Thomas *et al.*, 1997), rice (Cha *et al.*, 2002, Jiang *et al.*, 2004), Soybean (Luquez & Guiamet, 2002), Sorghum (Haussmann *et al.*, 2002), and durum wheat (Spano *et al.*, 2003). Stay green trait is known to be controlled by the recessive allele(s) (Cha *et al.*, 2002, Haussmann *et al.*, 2002, Luquez & Guiamét, 2002). Thomas *et al.* (1997) reported that two AFLP markers are closely linked to *sid* gene and useful for marker assisted selection in *Festulolium*. In *japonica*, rice stay green gene symbolized as *sgr*(t) was mapped to long arm of chromosome 9 through two RFLP markers (Cha *et al.*, 2002). QTLs mapping for stay green was developed with 128 markers in two sorghum recombinant inbred populations (Haussmann *et al.*, 2002).

In *Gramineae*, the tiller is one of many important factors determining the yield potential. However, the influence of maize tillers, which is probably introgressed from teosinte, has been controversial on grain yields. Livestock producers prefer multi-tillered corns for silage production because they had higher total yields at low population density than single stalked corns (Major, 1977). Choe *et al.* (1991, 1994) developed and evaluated tillering maize inbred and hybrids on agronomic characteristics for using as silage production. Recently Jang *et al* (2004) characterized two genes expressed in the crowns of tillers in multi-tillered maize.

AFLP method was known to be reliable and reproducible and generate larger molecular markers (Vos *et al.*, 1995). However, AFLP markers are difficult to directly use as a tool for plant breeding program because this method is relatively laborious, costly and complex Several researchers have reported conversion AFLP markers to sequenced tag site (STS) markers which is relatively simple to plant breeders (Qu *et al.*, 1998; Shan *et al.*, 1999; Seo *et al.*, 2001; Kim *et al.*, 2004). The objectives of this study were to identify AFLP markers to stay green trait and to develop sequence-specific PCR marker converted from AFLP markers in multi ears and tillers maize.

MATERIALS AND METHODS

Plant materials

A landrace of maize with multi ears and tillers (MET) was collected in Namwon, Korea. The plant was self-pollinated for six generations and was selected based on the number of

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tillers and ears/plant. The near-isogenic lines were grown in the field of Chungnam National University and visually scored with greenness of leaves after pollen shed. Each of the five plants scored as the highest or lowest for the greenness were collected and named as stay green MET or non-stay green MET, respectively.

AFLP analysis

Genomic DNA was isolated from leaf tissues using Plant DNA Isolation kit (Roche) according to manufactures instructions. AFLP analysis was conducted using AFLP Analysis System (InVitrogen) as described by Seo *et al.* (2001). Sixty four primer combinations were analyzed for selective amplification (AFLP Starter Primer Kit, InVitrogen). The selective PCR profile was as follows: ten cycles of 60 sec at 94 °C, 60 sec at an initial annealing temperature of 65 °C that was subsequently reduced by 1 °C for cycles two through ten, and 90 sec at 72 °C and then 33 cycles of 30 sec at 94 °C, 30 sec 56 °C, and 60 sec at 72 °C. The PCR products were evaluated on 2% agarose gel and then franctionated on 6% denaturing polyacylamide gels. The gels were stained using Silver Sequence DNA Staining Reagents (Promega)

Sequence analysis of polymorphic AFLP fragments

Polymorphic AFLP fragments were excised from polyacylamide gels using a razor blade. Each gel pieces was moved into a new tube containing 50 μl of distilled water and then heated to 100 °C for 10 min. The tubes were centrifuged briefly and the supernatant was transferred to each new tubes DNA fragments were sodium acetate/ethanol precipitated and dissolved with 10 μl of distilled water. The dissolved DNAs (5 μl) were used as a PCR template for reamplification. The DNAs were amplified with each of AFLP primer combinations. The PCR program was consisted of 60 sec at 94 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 56 °C for annealing, and 60 sec at 72 °C with a final extension cycle 72 for 4 min. Amplified PCR products were separated in 2% agarose gel and then eluted with the Nucleic Acid Purification Kit (Bioneer) The purified DNA fragments were cloned into pGEM-T Easy Vector Systems (Promega) and sequenced using BigDye Terminator Cycle Sequencing Ready Reaction kit with an ABI PRISM 3700 (Perkin-Elmer).

Conversion of AFLP markers to sequence tagged site (STS) markers

For conversion to STS markers, oligo primers were designed by sequences obtained from each of 5' or 3' sequences The sequences of STS primers and annealing temperatures were represented in Table 1. PCR reactions included 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, each 0.1 mM of dNTPs, each 0.5 μM of primers, 1 U of *Taq* polymerase, and 25 ng of genomic DNA. The PCR profile was, 4 min at 94 °C, and 35 cycles of 30 sec at 94 °C, 30 sec at 65 °C for annealing temperature, 60 sec at 72 °C with a final extension cycle 72 °C for 4 min. The PCR products were separated in 2% agarose gel

RESULTS

In order to develop molecular marker related to stay green phenotype, AFLP analysis was tested between MET corn with stay green trait and MET corn with non stay green trait (Fig 1). Total 64 primer combinations were evaluated for selective amplification of AFLP. Two primer combinations identified three reproducible polymorphic fragments in MET corn with stay green. E+AGC/M+CAC primer combination produced two specific polymorphic fragments linked to stay green trait. E+AAG/M+CAA primer combination amplified one polymorphic fragment. However, four primer combinations produced polymorphic fragments related in non-stay green trait. Each of E+AAC/M+CAC and E+ACT/M+CAC primer combinations amplified one specific fragment linked to stay green trait. E+ACG/M+CAC and

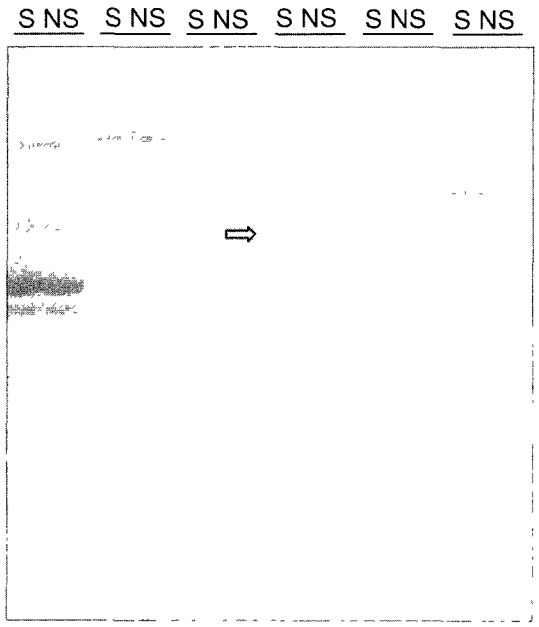

Fig. 1. Variation in AFLP patterns between MET maize with stay green trait and MET maize with non-stay green trait The arrow indicates polymorphic fragment stay green MET maize and non-stay green MET maize S: MET maize with stay green trait, NS. MET maize with non-stay green

Table 1. Profile of primer combinations which were used to detect polymorphism between MET maize with stay green trait and MET maize with non-stay green trait through AFLP analysis.

AFLP primer combination	Size (bp)	Entry of STS primer sets	Sequences of STS primer	Annealing temp (time)
E+AAG/M+CAA	266	MET1	5'-ACCATTCGGATATGCCTTGTTG-3 5'-CAACAAAGATGTTTTTGCTTGG-3	65 (30 sec)
E+AGC/M+CAC	204	MET2	5'-AGCGAGGGTTTCCGCAGGTAC-3 5'-CACAAGACGCTGGGCACTCAG-3	65 (30 sec)

E+ACG/M+CAG primer combinations produced two polymorphic fragments, respectively.

The two polymorphic fragments linked to stay green trait were cloned and then sequenced for conversion to STS markers. One fragment, which was amplified with E+AAG/M+CAA primer combinations, possessed 298 bp long and showed a 91% homology with maize retrotransposon *Cinful-1* (Accession no. T14595). One out of two polymorphic fragments produced with E+AGC/M+CAC primer combination had 236 bp long and matched a 96% homology with an intron region of 22 kDa alpha zein gene cluster in *Zea mays* (Accession no. AF090447) (data not shown).

Primers for STS analysis were designed from both end sequences of each two polymorphic fragments. Sequence specific primers, designed as MET1 and MET2, were synthesized 21 bp to 22 bp with 36% to 62% GC contents (Table 1). In order to assess the possibility for conversion AFLP to STS markers, PCR amplifications using each STS primer set were conducted using the template DNAs from stay green MET and non-stay green MET (Fig. 2). MET1 primer set amplified single monomorphic PCR products in the both MET types. However, one out of two fragment generated with MET2 primer set in the stay green MET was not produced in the non-stay green MET.

In order to explain the lack of conversion into STS marker in the case of MET1 primer set, PCR products derived from non-stay green MET were cloned, sequenced, and then compared with AFLP fragment sequences from stay green MET (Fig 3). The PCR products amplified with MET primer set was of 298 bp long, whose size is identical to that of AFLP fragment from stay green MET However, comparison of the both sequences represented in 2 bp sequence differences in internal sites.

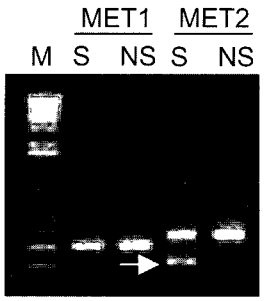

Fig. 2. PCR products amplified with MET1 and MET2 primer sets The arrow indicate polymorphic DNA fragment S· MET maize with stay green trait, NS MET maize with non-stay green M· 1 kbp molecular marker (InVitrogen)

DISCUSSION

The varieties of maize for silage have to possess high total yield including grain yield. The multi-tillered hybrids of

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 SG MET CAACAAAGATGTTTTTGCTTGGACAACCAATGATCTCTGTGGCGTCAATAGAGACATCAT  60
NSG MET CAACAAAGATGTTTTTGCTTGGACAACCAATGATCTCTGTGGCGTCAATAGAGACATCAT  60

          *
 SG MET TGAGCACTCACTCAATGTGGACCCATCCTTCAGACCAAGAAAGCAGAGGCTTCGGAAAAT 120
NSG MET TGAACACTCACTCAATGTGGACCCATCCTTCAGACCAAGAAAGCAGAGGCTTCGGAAAAT 120

                    *
 SG MET GTCCGATGATAAGGCTGAAGGTGCACGGAATGAAGTGAAGAGACTTCTAAGTGCCGACGT 180
NSG MET GTCCGATGATAAAGCTGAAGGTGCACGGAATGAAGTGAAGAGACTTCTAAGTGCCGACGT 180

 SG MET TATTAGAGAAGTAACATACCCAGAGTGGCTGGCCAACACTGTCATGGTAAAGAAAGCTAA  240
NSG MET TATTAGAGAAGTAACATACCCAGAGTGGCTGGCCAACACTGTCATGGTAAAGAAAGCTAA  240

 SG MET CAGAAAATGGAGAATGTGTATTGACTTTATAGATCTCAACAAGGCATATCCGAATGGT    298
NSG MET CAGAAAATGGAGAATGTGTATTGACTTTATAGATCTCAACAAGGCATATCCGAATGGT    298
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Fig. 3. Comparison of sequences of the PCR products amplified the MET1 primer set in MET maize with stay green trait and MET maize with non-stay green The asterisks indicate different sequences from two phenotypes

maize were known to possess high total yields than single stalked hybrids but lower grain contents (Major, 1977). However, multi-tillered hybrid developed by Choe *et al.* (1991, 1994) had higher fresh matter yields and dry matter yields than single stalked hybrids. Therefore, multi-tillered maize could be used as good stuff for silage by livestock producers.

We applied two marker systems to tagging stay green trait of MET maize. Thomas & Howarth (2000) classified five stay green phenotypes according to the progress of senescence. In the type A among five types senescence is delayed than wild type but proceeds at normal rate. The stay green MET maize showed similar response with type A (data not shown). Marker systems were developed in several crops for selecting and mapping of stay green trait. Thomas *et al.* (1997) reported two AFLP markers are closely linked to the *sid* gene in *Festulolium*. In rice the *sgr*(t) gene, which is controlled by a single recessive, was mapped to long arm of chromosome 9 between two RFLP markers (Cha *et al.*, 2002). The mapping of quantitative trait loci for stay green were constructed with 128 markers in two sorghum recombinant inbred populations (Haussmann *et al.*, 2002). In this study three AFLP markers were developed for stay green trait in multi-tiller maizes. Although we did not assign the relative locations/distances of two markers, they were expected to be useful as a tool for mapping with segregating populations and other molecular markers reported on loci of chromosomes. AFLP marker system was relatively laborious, costly, and complex for maize breeders. Therefore, it is necessary to convert AFLP markers to STS markers because simple PCR methods are required by plant breeders. Although several researchers have tried to convert AFLP markers to STS markers, they are not easily converted to simple PCR-based markers. Shan *et al.* (1999) reported that only 6 out of 26 AFLP markers are successful to amplify a fragment from the expected genome type. In our previous report one STS marker was successfully converted from 12 primer pairs derived from AFLP markers (Seo *et al.*, 2001). Seo *et al.* (2001) suggested that a possible reason for the lack of an efficient conversion might be that STS primers have lost the internal site of AFLP polymorphisms. In this report one STS primer set is failed to convert to STS marker from AFLP marker. As shown to compare sequences of PCR products derived from non-stay green MET with polymorphic AFLP fragment, both represented in 2 bp sequences differences in internal sites. In the previous report we observed similar results that only one bp of sequences of a fragment amplified with STS marker set is unmatched to sequences of AFLP polymorphic fragment (Seo *et al.*, 2001). Therefore, this result might support a possible reason for an inefficient conversion described by Seo *et al* (2001).

One STS primer set, MET2, is successful to amplify the specific polymorphic fragment linked to stay green trait although also to amplify the unexpected fragment. The STS marker might be used as an efficient tool for selection of multi-tillered maize with select stay green trait as well as AFLP markers.

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REFERENCES

Cha, K. -W., Y. J. Lee, H. -J. Koh, B. -M. Lee, Y. -W Nam, and N. -C. Paek. 2002. Isolation, characterization, and mapping of the stay green mutatnt in rice. Theor Appl. Genet. 104 526-532

Cheo, B. H., H. B Lee, and W K. Lee 1991. Agronomic characteristics of maize inbreds with tillers and their hybrids Korean J. Breed. 23 265-268

Cheo, B H , H B Lee, W. K Lee, H C. Ji, C. T. Chung, and S. K Jong. 1994 Characteristics of tillering maize inbred IK4. Korean J. Breed 26 447-450

Gentinetta E , D. Ceppi, C. Lpori, G Perico, M Motto, and F. Salamini 1986. A major gene for delayed senescence in maize Pattern of photosynthates accumulation and inheritance. Plant Breed. 97 193-203.

Haussmann, B. I. G , V. Mahalakshmi, B V .S. Reddy, N. Seetharama, C. T. Hash, and H. H. Geiger 2002 QTL mapping of stay green in two sorghum recombinant inbred populatons. Theor. Appl. Genet 106 : 133-142

Jiang G H , Y Q He, C G Xu, X. H Li, and Q Zhang 2004 The genetic basis of stay-green in rice analyzed in a population of double haploid lines derived form an *indica* by *japonica* cross. Theor. Appl. Genet 108 . 688-698

Jang, C. S., H. B. Lee, and Y. W. Seo. 2004. Molecular characterization of two genes, ZmRPS4 and ZmMET1, expressed in the crowns of tillers in maize Maydica (accepted)

Kim, D S , I S. Lee, C S Jang, S Y. Kang, H. S. Song, Y I. Lee, and Y W Seo. 2004 Development of AFLP-derived STS makers for the selection of 5-methyltryptophan resistant rice mutants Plant Cell Rep. 23 . 71-80

Luquez, V. M and J. J. Guiamét 2002. The stay green mutants *d1* and *d2* increase water stress susceptibility in soybeans. J Exp Bot. 53 . 1421-1428

Major D. J 1977 Seasonal dry-weight distribution of single-stalked and multi-tillered corn hybrids grown at three population densities in southern Alberta. Can J Plant Sci 57 . 1041-1047

Qu, L-J , T. N. Foote, M A. Roberts, T A. Money, L Aragon-Alcaide, J W. Snape, and G Moore 1998 A simple PCR-based method for scoring the *ph1b* deletion in wheat Theor. Appl Genet. 96 371-375

Seo, Y W , C S Jang, and J W Johnson. 2001 Development of

AFLP and STS markers for identifying wheat-rye translocations possessing 2RL. Euphytica 121 · 279-287

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

Spano, G., N. D Fonzo, C Perrotta, C Platani, G. Ronga, D W Lowlor, J. A Napier, and P R Shewry 2003 Physiological characterization of stay green mutants in durum wheat J. Exp Bot. 54 1415-1420.

Thomas H., C Evans, H M Thomas, M W. Humphreys, G Moran, B. Hauck, and I. Donnison 1997. Introgression, tagging and expression of a leaf senescence gene in Festulolium New Phytol. 137 . 29-34

Thomas H. and C. J. Howarth. 2000. Five ways to stay green J Exp Bot. 51 : 329-337.

Vos, P., R. Hongers, M. Bleeker, M. Reijans, T. van de Lee, M Hornes. A Frijters, J. Pot, J. Peleman, M Kuiper, and M Zabeau. 1995. AFLP a new technique for DNA fingerprinting Nucleic Acids Res 23 . 4404-4414.