ITEMS FROM INDIA

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Rapid identification of a hidden, co-migratory AP-PCR marker in wheat by band-stab PCR-RFLP.

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Abstract. Analysis of bread wheat genotypes for AP-PCR markers linked to rust resistance gene *Sr31* identified a ~580-bp band that showed high intensity ($SS30.2_{580(H)}$) among the resistant genotypes. Further analysis of this band by band-stab PCR followed by restriction analysis, revealed presence of two co-migratory bands, $SS30.2_{580(ad)}$ and $SS30.2_{580(L)}$. Band $SS30.2_{580(L)}$ was present among all the genotypes, irrespective of the status of the *Sr31* gene, whereas $SS30.2_{580(ad)}$ was present specifically among the *Sr31*-carrier genotypes. Band $SS30.2_{580(ad)}$ was identified from clones of $SS30.2_{580(H)}$, sequenced, and converted into a SCAR marker linked to the *Sr31* gene. The method reported here allows use of intensity difference of bands, which could be due to a hidden co-migratory band in PCR profiles for marker development.

Introduction. Molecular markers are useful for MAS in breeding of crop plants (Tanksley et al. 1989). A large number of markers for various traits have been reported for several crops in the last decade (Mohan et al. 1997). In wheat, molecular markers such as RFLP, RAPD, STS, SCAR, and AFLP have been reported for a number of traits (Gupta et al. 1999 and MASwheat web site http://maswheat.ucdavis.edu). Arbitrary primers (as in RAPD, DAF, and AP-PCR) are commonly used for developing DNA markers. The marker for MAS should be easy to score, highly reproducible, and should be specific. Hence, a marker identified by the above-mentioned methods is generally converted into SCAR marker before its use for MAS (Paran and Michelmore 1993).

While scoring DNA markers, generally the bands that show unambiguous polymorphism are considered, whereas differences in the intensity of bands among the parents and/or accessions are ignored. Intensity differences among bands are generally observed among accessions and is likely to be due to small variations in the amount of genomic DNA or PCR per se or both. We present data to show that among wheat accessions where polymorphism is low, even a difference in intensity of band should be analyzed. We describe a method to identify and characterize bands that show difference in intensity, due to the presence of more than one co-migratory band. The method involves band-stab PCR and restriction analysis in which a hidden co-migratory band present within a high intensity band could be identified and characterized.

Materials and Methods. DNA was isolated from wheat leaves according to Krishna and Jawali (1997). Arbitrarily Primed-PCR (AP-PCR) was performed using long primers (>15 mer) as described by Saini et al. (2004). Amplification products were resolved on 2% agarose gel in 1 X TBE buffer, stained with ethidium bromide and visualized under UV light and photographed.

Selective reamplification of the DNA band of interest from the agarose gel was carried out by band-stab method (Bjourson and Cooper 1992). Agarose gels were stained with ethidium bromide and DNA fragments were visualized on a UV transilluminator. The DNA band of interest was stabbed with a sterile needle (22 gauge), briefly dipped into a PCR tube containing 25 µl reaction mix and subjected to PCR amplification using same primer and thermal cycling conditions.

The band-stab amplified products (500 ng) were digested by 10 restriction enzymes (Bangalore Genei Pvt. Ltd., India) including *Taq*I and *Alu*I in a total reaction volume of 20 µl, according to the conditions specified by the manufacturers. The digested products were analysed on a 2.5% agarose gel as explained above.

The band-stab PCR products were polished using Vent DNA polymerase (Exo+: New England BioLabs Inc., Beverley, MA, USA.) and ligated into vector plasmid Bluescript at the *EcoR*V site using the Rapid DNA ligation kit (Ro-

che Molecular Biochemicals, Germany). Transformation of E. coli (DH5α) cells with ligation product and identification of the colonies carrying recombinant plasmid was done according to standard protocols (Sambrook et al. 1989).

The insert DNA from the recombinant plasmid was amplified by colony PCR as follows. Colonies carrying the recombinant plasmid were stabbed with a fine sterile needle tip and briefly immersed into a PCR tube containing 25-µl PCR reaction mixture and plasmid specific primers M13F (5′ CGACGTTGTAAAACGACGGCCAGT 3′) and M13R (5′ CACAC-AGGAAACAGCTATGACCATG 3′). PCR amplification was carried out under the following cycling conditions: 95ºC–5 min, 60ºC–1 min, 72ºC–1.5 min (one cycle); 35 cycles of 95ºC–1 min, 60ºC–1 min and 72ºC–1.5 min followed by final extension of 72ºC–10 min. Where mentioned, the colony PCR product was subjected to restriction analysis.

Results and Discussion. From the AP-PCR profile of 45 wheat genotypes, a ~580-bp band with high intensity (designated as SS30.2580(H)) was found to be present only among genotypes carrying *Sr31* (Fig.1A), and a band of similar size with low intensity (designated as $SS30.2_{580(L)}$) was detected among the genotypes not carrying *Sr31*.

Whether $SS30.2_{580(H)}$ and $SS30.2_{580(L)}$ were a) same sequence amplified to different extent, b) two different sequences of similar size, c) mixture of different sequences was investigated. Band $SS30.2_{580(H)}$ from PBW-343 (an *Sr31* carrier) and $SS30.2_{580(L)}$ from Kalyansona (a noncarrier of *Sr31*) were amplified individually by band-stab PCR method. Because the products were indistinguishable on the basis size (Fig. 1B, lanes 3 and 4), they were digested with restriction enzymes and the profiles were analyzed. Results showed that profiles obtained after digestion with *Taq*I and *Alu*I, could distinguish the two products. The profiles of the two bands after digestion with *Taq*I is shown in Fig. 1B (lanes 5 and 6). The $SS30.2_{580(L)}$ consisted of a single DNA band that yielded fragments 'b'(~360 bp), and 'c' (~220 bp), whereas $SS30.2_{580(H)}$ was a mixture of two DNA bands that yielded four fragments, 'a' (~420 bp), 'b' (~360 bp) 'c' (~220 bp), and 'd' (~160 bp). Comparison of the two profiles showed that $SS30.2_{580(H)}$ band consisted of two bands, i.e., $SS30.2_{580(L)}$ consisting of fragments 'b' and 'c' and a second band designated as $SS30.2_{580(ad)}$ consisting of fragments 'a' and 'd'. These results suggested that the DNA fragment $SS30.2_{580(ad)}$ may be associated with *Sr31* gene.

To confirm the above findings, $SS30.2_{580(H)}$ and $SS30.2_{580(L)}$, bands from the remaining 42 wheat cultivars were amplified by band-stab PCR and the

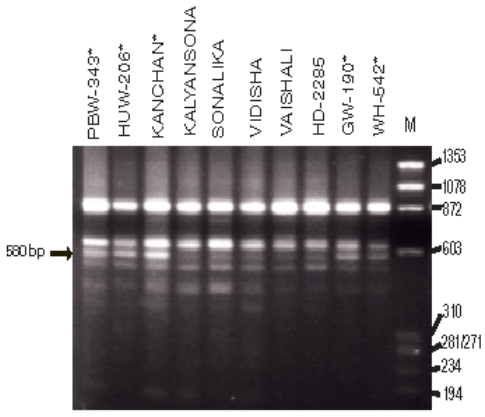

Fig. 1A

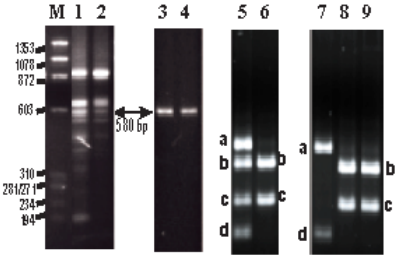

Fig. 1B

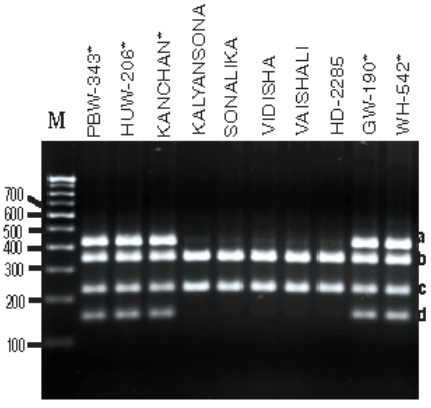

Fig. 1C

Fig. 1A. AP-PCR profiles of wheat genotypes obtained using primer SS30.2. The names of the genotypes are shown on top of the lanes. An '*' indicates genotypes carrying *Sr31* gene. An arrow indicates the position of ~580 bp band. Lane 'M' indicates molecular size marker (PhiX174 digested with *Hae*III).

Fig. 1B. Analysis of band stab-amplified ~580-bp bands from resistant and susceptible genotypes: Lanes 1 and 2, AP-PCR profiles of resistant and susceptible genotypes using SS30.2 primer; Lanes 3 and 4, band stab-amplified 580-bp bands from resistant and susceptible genotypes; Lane 5, restriction profile of $SS30.2_{580(H)}$ fragment by *Taq*I; Lane 6, restriction profile of $SS30.2_{580(L)}$ by *Taq*I; Lanes 7 and 8, *Taq*I restriction profiles of two different types of clones of 580 bp from resistance genotype; Lane 9, *Taq*I restriction profile of single type clone of 580 bp from susceptible genotype.

Fig. 1C. *Taq*I restriction profiles of band-stab amplified 580-bp band from wheat cultivars (carrier and noncarrier of *Sr31*). An '*' indicates genotypes carrying *Sr31* gene. Lane 'M' indicates the 100-bp DNA

products obtained were subjected to *Taq*I analysis. As an example, the profile obtained from a few cultivars is shown in Fig. 1C (p. 55). The results showed that both $SS30.2_{580(ad)}$ and $SS30.2_{580(L)}$ were present among all *Sr31* carriers, whereas only $SS30.2_{580(L)}$ was present among the noncarriers of Sr31, establishing that $SS30.2_{580(ad)}$ is associated with *Sr31*.

To further establish that the $SS30.2_{580(H)}$ contains $SS30.2_{580(ad)}$ and $SS30.2_{580(L)}$, and $SS30.2_{580(L)}$ is a single fragment, the band-stab PCR amplified products were cloned and the insert DNA from several clones were analyzed as mentioned in materials and methods using *Taq*I. The analysis revealed that the clones obtained from $SS30.2_{580(H)}$ band consisted of two types: inserts $SS30.2_{580(ad)}$ and $SS30.2_{580(L)}$ that yielded either fragments 'a' and 'd' (Fig. 1B, lane 7, p. 55) or 'b' and 'c' (Fig. 1B, lane 8, p. 55), whereas $SS30.2_{580(L)}$ clones carried one type of insert that yielded fragments 'b' and 'c' (Fig. 1B, lane 9, p. 55). These results confirmed that $SS30.2_{580(H)}$ consisted of two bands of same size of which $SS30.2_{580(ad)}$ was associated with *Sr31*. Furthermore, the $SS30.2_{580(ad)}$ band has been sequenced, and SCAR primers designed and validated (Das et al. 2006).

In this paper we have described a method for identification and characterization of a band that is polymorphic due to differing intensity. This method allows the quick identification of the sequence dissimilarity between two similar sized bands or a band without the use of time consuming Southern hybridization technique that is generally needed for such analyses. This study stresses the need for careful analysis of DNA marker profiles for intensity differences, while looking for markers linked to traits of interest particularly among the crop species such as wheat in which the diversity is poor.

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Current activities: Improvement of wheat quality and rust resistance in Indian wheat.

B.K. Das and S.G. Bhagwat (Nuclear Agriculture & Biotechnology Division).

Improvement of wheat for quality in Indian wheat background is being carried out using HMW-glutenin subunits as a selection criterion. Rust-resistance genes such as *Sr31*/*Lr26*/*Yr9*, *Sr26*, and *Sr24*/*Lr24* are being combined with high yielding ability and protein subunits for quality traits. Progenies from several intervarietal crosses, in different generations such as F_2, F_3, and F_4 were sown and selections are being developed.

Marker-assisted selection for rust resistance genes and quality related genes.

B.K. Das and S.G. Bhagwat (Nuclear Agriculture & Biotechnology Division).

Marker-assisted breeding for combing *Sr24*, *Sr31*, and *Glu-D1d* (coding for HMW-glutenin subunits 5+10) was carried out in intervarietal crosses. In the F_2, 174 plants were analyzed by SCAR markers. Plants carrying three genes were selected and will be advanced further.

For transferring *Sr24* and *Glu-D1d* into the good cultivar HD-2189, backcross breeding was adopted. Twenty-two BC_2F_1 plants were grown and, DNA from leaves of four weeks old individual plants were extracted and screened by SCAR markers for these two genes. Two plants carrying both the genes were identified and backcrossed with the recurrent parent HD-2189.

Assessment of allelic variation with microsatellite marker Xgwm261 and its effects on agronomic traits in heat stress and nonstressed locations in Indian wheat cultivars.

Suman Sud and S.G. Bhagwat (Nuclear Agricultural and Biotechnology Division).

The need for genes/alleles in wheat cultivars to improve their performance in heat-stress environments is urgent. The gene *Rht8* is reported to confer tolerance to heat stress. Allelic variation was analyzed with microsatellite marker *Xgwm261* in wheat cultivars. The analyses showed that 165-bp and192-bp alleles were more frequent as compared to 174-bp allele. The 192-bp allele, which is supposed to be associated with *Rht8* gene, is present in 45% of the tested cultivars. All the tested cultivars were grown in heat-stress and nonstressed locations for assessing their allelic advantage in respective environments. Cultivars with the 192-bp allele did not show significant height reduction over the 165-bp and 174-bp alleles in both environments, indicating presence of height neutral 192-bp allele. Although pedigree analyses did indicate involvement of Akakomughi, the donor of *Rht8* gene in the pedigrees of Indian cultivars, the 192-bp fragment that was found in the Indian wheat cultivars was not the one associated with *Rht8* from Akakomughi. The 192-bp fragment appeared to be the one that is contributed by 'Norin10 / Brevor' lines. The parentage information from literature, molecular analyses, and agronomic data in the present study indicated absence of *Rht8* gene in Indian cultivars.

Callus induction and regeneration in wheat.

Chun Mei Chang, P. Suprasanna, and S.G. Bhagwat (Nuclear Agriculture & Biotechnology Division).

Callus induction was attempted from different tissue explants such as nodes, immature rachis, and mature and immature embryos of the wheat cultivars HD2189 and Unnath C306. A MS medium containing 2 and 4 mg/L 2-4D along with casein hydrolysate was found to be favorable for high frequency callus induction. Regenerating calli were obtained only from immature embryos. The regenerated plants were hardened and transplanted to soil. The regenerants showed normal plant morphology and grain filling.

Publications.

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