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DNA polymorphism in *Cab* locus of tomato induced by tissue culture

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Plants were regenerated from callus induced from leaf disc explants of a tomato F_1 hybrid heterozygous for three marker loci anthocyaninless (*a*), without anthocyanin (*aw*), and hairless (*hl*). Regenerants were studied for somaclonal variation at the phenotypic level by scoring for variation in the marker loci, and at the DNA level by probing geomic DNA blots with a chlorophyll a/b binding protein (*Cab*-3C) cDNA sequence. While no variation was observed at the phenotypic level in over 950 somaclones studied, DNA polymorphism for the *Cab* locus could be detected in two out of 17 somaclones tested. Tissue culture induced variation at the phenotypic level for specific loci is very low (less than 0.001 for *a*, *aw* or *hl*) but DNA sequence changes are induced at much greater frequency (~ 0.1 for a multicopy gene family such as *Cab*).

The phenomenon of somaclonal variation, has been documented in a variety of crop plants[1]. It has been demonstrated that several nuclear and cytoplasmically inherited changes are related to alterations in the DNA sequence or the methylation status of the somaclone. The expression of a variant ADH 1 isozyme in a maize somaclone was traced to a single base substitution resulting in a consequent amino acid substitution in the isozyme[2]. Similarly, reduction in r DNA units correlated with heritable and reduced C-banding in the NOR region was found in a family of triticale somaclones[3]. Alteration in the organization of intergenic spacers of r RNA genes has been reported in somaclones of wheat[4], barley[5] and potato[6]. Changes in the expression of cytoplasmically inherited traits such as male sterility and sensitivity to *Drechslera maydis* toxin in maize somaclones has been associated with deletions and rearrangements in the mitochondrial DNA[7]. Brown[8] reported that phenotypically variant somaclones of maize often showed significant alterations in the methylation status of both housekeeping and structural genes.

In this paper we report the detection of variation in the chlorophyll a/b binding protein (*Cab*-3C) locus in tomato somaclones.

Materials and Methods

Tissue culture and plant regeneration—A F_1 hybrid of tomato was obtained by crossing two genetic marker stocks 19-(1B) and GC-1, seeds of which were a gift from M.S. Ramanna, University of Wageningen, Netherlands. The stock 19-(1B) has the genetic constitution (*aw/aw*; *A-Hl/A-Hl*) i.e., it is recessive for the chromosome 2 marker without anthocyanin (*aw*) and dominant for the markers anthocyaninless (*a*) and hairless (*hl*) both on chromosome 11, and is phenotypically green and hairy. The other stock GC-1, is dominant for *aw* marker and recessive for *a* and *hl* markers (*Aw/Aw*; *a-hl/a-hl*) and is phenotypically green and hairless. The F_1 hybrid had the genotypic constitution *Aw/aw*; *A-Hl/a-hl* and was phenotypically hairy besides being purple due to complementation between the dominant alleles at the *a* and *aw* loci.

Leaf disc explants from a F_1 grown under aseptic conditions were induced to callus on medium consisting of inorganic constituents of MS[9], B_5[10] vitamins (10 mg/l thiamine, 1 mg/l niacin, 1 mg/l pyridoxine), 100 mg/l ascorbic acid, 2% sucrose and the growth hormones BAP 2 mg/l and IBA 0.4 mg/l. The medium was gelled with 0.8 bacteriological grade agar after adjusting the *p*H to 5.8. Cultures were maintained on a 18 hr day/16 hr night light regime at 25° ± 2° C. Callus initiated from leaf discs was transferred to and maintained on MS medium with B_5 vitamins, 100 mg/l ascorbic acid, 2% sucrose and 0.8 mg/l BAP. Shoots regenerated from the callus were transferred to rooting medium consisting of MS with 1% sucrose and 0.1 mg/l NAA. Well rooted plants were washed free of agar and kept in a humid chamber for 1-2 weeks in culture vials with the roots submerged in tap water. Plantlets were scored at this stage for anthocyanin pigmentation and hairyness to detect changes, if any, at the three specific marker loci *aw*, *a* and *hl*. Since the source of the explants, the F_1 hybrid, is heterozygous dominant, a tissue

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culture induced change from dominant to recessive would be detected as loss of hairyness or anthocyanin pigmentation in the somaclones. Some of the plantlets were transferred to the field in order to obtain leaves for DNA extraction.

DNA extraction—For DNA isolation, young expanding leaves were harvested from each regenerant, frozen in liquid nitrogen and stored at −70°C. Total genomic DNA was isolated using the protocol of Kochert *et al*[11]. Five to 10 g of leaves were ground to a fine powder in liquid nitrogen and suspended in 25 ml DNA extraction buffer consisting of 7 *M* urea, 0.35 *M* NaCl, 0.05 *M* Tris (*p*H 8.0), 0.02 *M* EDTA (*p*H 8.0), 2% Sarkosyl and 50 ml/l phenol reagent. The cells were lysed at 60°C for 10 min in 0.75 ml of 20% SDS. The mixture was emulsified by gentle shaking in 15 ml of 24:1 chloroform:isoamyl alcohol for 15 min. Phase separation was achieved by centrifugation and nucleic acid precipitated from the aqueous phase with 0.6 volume isopropanol. The crude DNA was spooled out, washed with 70% ethanol and resuspended in 4 ml TE. DNase free RNase was added to a final concentration of 10 μg/ml and the sample incubated at room temperature for 10 min. The DNA was then precipitated with 2 volumes ethanol and spooled out, washed in 75% ethanol, dried and dissolved in 0.5 ml TE.

Southern blotting and hybridization—DNA isolated by the above protocol was digested with EcoRI (Promega) using 15 μg DNA/lane and 5 units enzyme/μg DNA. The restriction mixture was incubated at 37°C for 10-12 hr. The restricted DNA samples were electrophoresed in 0.8% agarose gel in 0.5 × TBE buffer.

Prior to blotting, the DNA in the gel was depurinated by treatment with 0.25 *N* HCl for 10 min. The gel was rinsed with water and denatured by gentle agitation in 0.5 *M* NaOH, 0.5 *M* NaCl for 60 min. The DNA was blotted, using 0.5 *M* NaOH, 0.5 *M* NaCl as transfer buffer, onto a Genescreen plus (Dupont) nylon membrane soaked in 0.5 *M* NaOH, 0.5 *M* NaCl, employing a vacuum blot apparatus. The DNA blotted membrane filter was washed in 0.2 *M* Tris HCl, (*p*H 7.5), 2 × SSC for 10 min, dried, and baked at 80°C for 2 hr in a vacuum oven.

The baked nylon membranes were prehybridized for about 8 hr at 65°C in buffer containing 5% Dextran sulphate, 5 × SSC, 0.05 *M* sodium phosphate buffer, 2.5 × Denhardt's solution, 0.0025 *M* EDTA, 0.6% SDS and 100 μg/ml sonicated and denatured salmon sperm DNA. About 0.2 ml of prehybridization fluid was used per square centimeter of membrane.

The Southern blots were probed with a cDNA clone of chlorophyll a/b binding protein *Cab*-3C sequence[12], from E. Pichersky, University of Michigan. For use as probe, 1 μg of whole plasmid DNA containing the cloned *Cab*-3C sequence was made radioactive by incorporation of ^{32}P-dCTP using a Dupont nick-translation kit. The probe was freed of unincorporated nucleotides by passing through a sephadex G-50 column. For hybridization with the denatured probe DNA the buffer volume was reduced to 0.05 ml/cm^2. Hybridization was done at 65°C in a water bath for 20-24 hr.

The hybridized membranes were washed free of non-specifically bound probe by agitating in 2 × SSC for half an hour at 65°C followed by a more stringent wash in 2 × SSC, 0.1% SDS at 65°C for 10-15 min.

Autoradiography of the hybridized membranes was done using Kodak X-ray films.

Results and Discussion

A total of 983 plants were regenerated and studied for somaclonal variation. None of the somaclones showed any change with respect to the marker loci *a*, *aw* and *hl* and did not differ in their morphology from the F_1 plant from which leaf explants had been taken (hence forth referred to as the F_1 donor plant).

Total genomic DNA isolated from the two parental genetic stocks, the F_1 donor plant and 17 randomly chosen phenotypically similar somaclones when restricted with EcoRI and probed with the *Cab*-3C cDNA clone showed that the two parental stocks, the F_1 donor and 15 out of the 17 somaclones had the same restriction pattern. Polymorphism was observed in two of the 17 somaclones tested (Fig. 1). In one somaclone a major band corresponding to a 23 kb fragment was missing (lane SC 2 in Fig. 1), while in the other, only a faint band could be detected at the same position (lane SC 3 in Fig. 1).

The phenotypic manifestation of the DNA sequence alteration in the variant somaclones was probably absent due to the multicopy nature of the CAB genes. The chlorophyll a/b binding proteins in higher plants are coded by a multicopy nuclear gene family[13,14,15]. There are five such loci in tomato of which *Cab*-2, *Cab*-4 and *Cab*-5 have a single gene each while *Cab*-1 and *Cab*-3 are complex. The *Cab*-1 locus has 4 genes and *Cab*-3 locus has 4 or 5. Within each of these two loci, the homology is 92% and between genes of the two clusters it is 88%. Using the *Cab*-3C probe, under the stringency conditions employed in the present investigation, it was not possible to differentiate between *Cab*-1 and

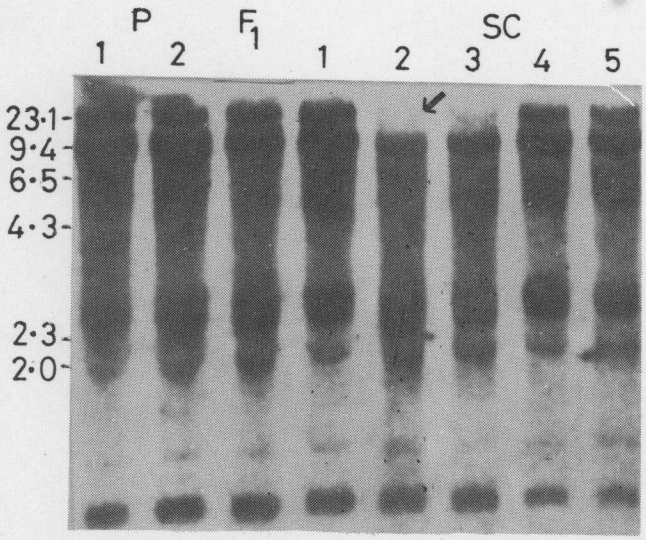

Fig. 1—Southern blot of EcoRI digested genomic DNA probed with *Cab*-3C cDNA. [Lane 1 and 2: Parental genetic stocks 19-(1B) and GC-1 respectively (see text for description). Lane 3: F_1 donor plant. Lane 4-8: Somaclones 1 to 5. Arrow indicates missing band. Molecular weight markers at left in Kb corresponding to Lambda-HindIII digested DNA]

Cab-3 loci, the sequence change may have occurred in either.

In the two variant somaclones, the absence of a band was not associated with shift in the position or intensity of any other band in the lane. The polymorphism therefore probably represents a deletion rather than a base substitution or insertion. Although the size of the DNA fragment, corresponding to the missing band, is about 23 kb, the deletion may not necessarily include the whole length of the fragment but would nevertheless be atleast the size of the *Cab*-3C probe. The complete absence of a single band represents a homozygous deletion while a faint band at the normal position in the other somaclone indicates a heterozygous deletion. For confirmation of this hypothesis, RFLP analysis of the progeny of the variants is in progress.

Our results demonstrate that while somaclonal variation at the phenotypic level may be very low (no variant obtained in 983 somaclones scored i.e., less than 0.001 for the marker loci *a*, *aw* and *hl* tested in this experiment), the frequency of DNA sequence changes is many fold higher (2/17, i.e. 0.1 for a multicopy gene family such as *Cab*). With judicious use of suitable probes, the RFLP technique can help in the detection of sequence changes in genes governing complex traits, the screening or selection for which is otherwise difficult.

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