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Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation

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Abstract. Anther culture and *Hordeum bulbosum*-derived doubled haploid (DH) lines of barley (*Hordeum vulgare* L.) were analyzed for RFLP and RAPD polymorphisms. Polymorphisms were not detected in the anther culture- or *H. bulbosum*-derived DH lines among 273 RFLP and 89 polymerase chain reaction (PCR)-amplified DNA fragments assayed. It was calculated that base substitution or small deletion/insertion mutations had not been induced among 401 640 bp screened. Large deletion/insertion mutations were not observed among 33 Mb screened. Polymorphisms were observed when DNA was digested with the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I: these RFLPs originated primarily from the anther culture-derived doubled haploids. The data indicate that heritable DNA methylation changes had occurred during DH production, particularly with the anther culture method.

Key words: Anther culture – *Hordeum bulbosum* method – Doubled haploid – Mutation frequency – DNA methylation

Introduction

Doubled haploid (DH) lines are used for plant breeding, genetic analyses and to construct molecular marker maps (Kasha and Reinbergs 1982; Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993). In barley (*Hordeum vulgare* L.), DHs can be produced by *H. bulbosum* (Hb) and anther culture (AC) methods. Both techniques require an in vitro culture phase, but they differ in several aspects. (1) Length of time in culture: 3 weeks for Hb versus 2 months for AC. (2) Composition of the media: growth hormones are not used for the development of haploid embryos in the Hb technique while an auxin, indole acetic acid or naphthalene acetic acid, and a cytokinin, kinetin or benzylaminopurine, are commonly used in AC (for review see Pickering and Devaux 1992). (3) Regeneration mechanism: plants are obtained through the development of zygotic embryos in the Hb technique but via organogenic and embryogenic structures in AC.

It is well established that the in vitro culture phase can induce genetic and cytoplasmic changes in regenerated plants (Larkin and Scowcroft 1981; Evans and Sharp 1983; Day and Ellis 1985; Pickering 1989; Ullrich et al. 1991). Moreover the conditions of the culture phase influence the frequency of these variations (for review see Karp 1991). Phenotypic changes such as albinism, variation in ploidy level, and chromosomal abnormalities have been reported in AC-derived regenerants (Clapham 1973; Mix et al. 1978; De Buyser et al. 1985; Charmet et al. 1986). Most of the gametoclonal variations negatively affect the agronomic performance of DHs (Baenziger et al. 1983; Powell et al. 1984; Snape et al. 1988; Marburger and Jauhar 1989). Snape et al. (1988) reported variation in Hb-derived DHs of wheat for three quantitative traits but did not find variation in barley Hb-derived DHs among seven quantitative characters studied.

Although genetic alterations induced by AC and Hb methods are well documented, there are very few reports on gametoclonal variation at the DNA level. Day and Ellis (1985) showed changes in the chloroplast DNA of wheat and barley albino plants derived by the AC method. Reports on genomic DNA are limited to the analysis of repetitive sequences (De Paepe et al. 1983; Rode et al. 1987). The purpose of this investigation was to assess the level of DNA variation in phenotypically normal DH barley plants derived by the Hb and AC techniques from one cultivar. Two classes of molecular markers were

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applied [restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD)] to analyze for base substitution and insertion/deletion events.

Materials and methods

Plant material. Seeds of the winter barley cultivar Igri were kindly provided by Mr. Darrozés (Serasem – Pérenchies, France). Haploid and spontaneous DH plants were produced through AC and Hb techniques according to Devaux (1987); the haploid plants were treated with colchicine to induce chromosome doubling (Pickering 1980). Before anthesis, all spikes were bagged to avoid cross pollination. At maturity, grains were collected from spontaneous and colchicine-treated DH plants and propagated in a field nursery. Two to four spikes per DH were bagged and collected at maturity. Five grains each of 60 randomly chosen DHs (30 from AC and 30 from Hb techniques) as well as seeds from the original lot of Igri were sown in a greenhouse. Three to four weeks later, the aerial parts of the seedlings were harvested, plunged into liquid nitrogen and lyophilized. Dried tissues were stored at $-20°$ C until DNA extraction.

DNA extraction. Genomic DNA was isolated as described in Kleinhofs et al. (1993). Briefly, total DNA was extracted from powdered lyophilized tissues using the cetyltrimethylammonium bromide and phenol-chloroform procedure and precipitated with 2-propanol. After dissolution in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8), the DNA was treated with RNAase A, extracted with chloroform, precipitated with ethanol and finally dissolved in distilled water at about 0.5 µg/µl.

RFLPs. About 10 µg of genomic DNA were digested with 30 units of restriction enzyme according to the manufacturer's recommendations. The 6 bp recognition site enzymes *Bam*HI, *Eco*RI and *Hin*dIII and the 4 bp recognition site enzymes, *Hpa*II and *Msp*I, were used. The latter two enzymes are isochizomers, but respond differently to methylation of the second C in the 5′-CCGG-3′ recognition sequence. Restricted DNA fragments were separated by electrophoresis and transferred from the gels to MSI (Micron Separations, Westborough, MA) membranes. The probes Adh1, Amy2, Cab, Chs, Glb1, Hor1, Isa1, Mub2, Pox, ABG307, BCD351, BG123, SBC218, pHv294T, Akp6, ksuA1, ksuA3, ksuF2, 21B9, and 25H2 were used to detect polymorphisms with the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII. The probes ABG471, ABC174, BCD351, BG123, ksuA3, 21B9, and 25H2 were used to detect polymorphisms with the restriction enzymes *Hpa*II and *Msp*I. The probes are from various sources described in Kleinhofs et al. (1993).

RAPDs. About 50 ng of genomic DNA were used as template for amplification with 11 random decamer primers as described by Kleinhofs et al. (1993).

Results

Assessment of mutation

Polymorphisms were not detected between the AC and Hb-derived DHs among 273 RFLP fragments revealed by 18 probes and using 3 restriction enzymes. The patterns obtained were identical to those of cv Igri. This indicates that base-substitution mutations, insertions, and deletions were not detected. The total number of base pairs (bp) screened for base substitutions and small (a few bp) insertion/deletion events was 294 840 and corresponds to the product of: (1) the number of DHs investigated – 60; (2) the total number of restriction fragments screened – 273; and (3) the number of bp screened per fragment – 18 [this corresponds to the 6 bp at the two ends of each fragment screened for loss of 6-base recognition sites plus the mean number of bp in a ca. 4 kb fragment screened for mutation to create the same restriction site (Kilian and Gale 1991)].

To estimate the number of bp screened for large insertions/deletions (detectable by agarose gels used to separate restriction fragments) a value of 4096 should be used instead of 18 in the above formula. This number corresponds to an expected average size of RFLP fragment generated by restriction enzymes with 6 bp recognition sites and gives a value of ca. 60 Mb analyzed for detectable insertions/deletions. This value was reduced by a factor of 2 to 30 Mb to correct for overlapping fragments, which were produced by different enzymes and detected by the same probe; the estimate is therefore likely to be conservative. In addition, no variation in fragment number, size, or intensity of hybridization was found using the ribosomal DNA intergeneric spacer probe, pHv294T (Gerlach and Bedbrook 1979) or the telomeric probe, Akp6 (Kilian and Kleinhofs 1992).

All electrophoretic patterns of PCR-amplified DNA fragments of the 60 DHs and cv Igri were identical, indicating that no mutation had occurred in annealing sites of the primers. The number of bp checked for nucleotide substitutions and small insertions/deleitons was 106 800. This number is based on the number of DHs

Table 1. Polymerase chain reaction (PCR)-amplified bands detected with 11 decamer primers used in this study

Primer	Sequence	No. of bands
CS19	5′-TAC GGC TGG C-3′	10
CS21	5′-CCC TAC CGA C-3′	8
CS31	5′-CTC GAC ACT G-3′	9
CS34	5′-GAT AGC CGA C-3′	9
CS35	5′-AGT CGC TCA T-3′	9
CS42	5′-CCC AGA ACA C-3′	8
CS45	5′-CAC GTC GGA G-3′	9
CS47	5′-TTG CCG TGT T-3′	8
CS50	5′-CCC AAA CTA G-3′	7
I04	5′-CCG CCT AGT C-3′	6
L20	5′-TGG TGG ACC A-3′	6
Total		89

investigated (60) multiplied by 20 (i.e. 2×10 bases) and by the 89 fragments screened (Table 1). The molecular basis for RAPD polymorphism is still obscure (Yu et al. 1993), so it is difficult to estimate the number of bp scanned for mutation using this technique. Since there is indirect evidence suggesting that a single bp mismatch between primer and template may result in a lack of product amplification (Williams et al. 1990), we used a value of 20 bp as a multiplication factor (2×10 bp, each annealing site) to calculate the number of bp screened for mutation. It is possible that it is an overestimate for loss of the RAPD band but, on the other hand, we did not take into account amplification of a new product due to a mutation event. If an average RAPD band size (600 bp) is used as a multiplication factor (instead of 20 bp) the formula gives ca. 3 Mb of DNA analyzed for insertion/deletion events.

Frequency of mutation

From these experiments, we conclude that no mutation had occurred in 401 640 (294 840 by RFLPs and 106 800 by RAPDs) bp screened for small insertion/deletion events and nucleotide substitutions. Since the barley genome is approximately 5.5×10^9 bp (Bennett and Smith 1976), this represents 0.0073% of the barley genome scored for point mutations. Since no mutations were found in 401 640 bp that were screened, the frequency of mutation per bp due to the in vitro culture is less than 0.25×10^{-5}. There were no large insetions/deletions detected in ca. 33 Mb screened, corresponding to more than 0.5% of the barley genome.

Changes in DNA methylation

DNA digested with the methylation-sensitive restriction enzyme *Hpa*II yielded RFLPs, suggesting that DNA methylation changes had occurred during DH production (Fig. 1A and B). These RFLPs originated primarily from AC-derived DHs: 49 aut of 51 variable fragments detected with seven probes. Most of the variations were confirmed using the *Hpa*II isochizomer, *Msp*I. The recognition sequence 5′-CCGG-3′ is cleaved by *Msp*I when the internal C is methylated, but not if the 5′-C is methylated, while the isochizomer *Hpa*II does not cleave when either C is methylated. Thus in most cases methylation had occurred at least at the 5′-C in the recognition sequence.

Methylation polymorphisms appear to be randomly distributed among the DHs, but nonrandomly distributed among the probes. Most of the 30 AC-derived DHs showed polymorphism with at least one probe, but only three DHs were different from the control with all three of the probes accounting for most of the observed polymorphisms. These probes, BCD351, ksuA3, and 21B9, revealed 48 out of the 49 RFLPs in the Ac-derived DHs suggesting nonrandom distribution of methylation changes among the probes. Polymorphisms were due to

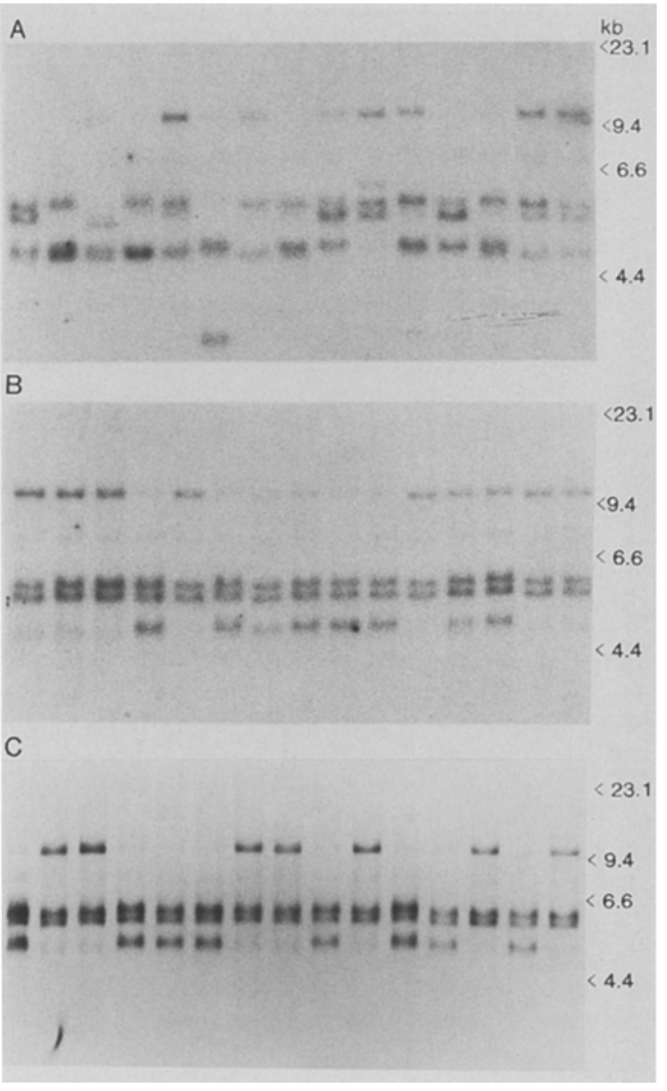

Fig. 1A–C. Doubled haploid- and individual seed-derived cv Igri line DNA digested with *Hpa*II and hybridized with probe 21B9. **A** Anther culture-derived doubled haploids (DHs); **B** *Hordeum bulbosum*-derived DHs; **C** Seed-derived lines. Note the large variation in the size and intensity of the individual bands in the anther culture-derived doubled haploids (**A**) and the distinct segregation pattern for the highest and lowest molecular weight bands in the seed-derived lines (**C**). The *H. bulbosum*-derived doubled haploids (**B**) show some variation in the hybridization intensity of the highest and lowest mol. wt. bands

increased methylation (37 out of 51 cases) or decreased methylation (14 out of 51 cases), as indicated by higher or lower molecular weight DNA fragments hybridizing, respectively.

To determine whether DNA methylation heterogeneity existed in the original lot of Igri, the DNA of 16 individual plants was digested with *Hpa*II and hybridized with the probes BCD351, ksuA3, 21B9, and BG123. Polymorphisms were not found among these 16 plants except with the probe 21B9, which hybridized with three fragments in all lines (Fig. 1C). Two bands were identical among all plants tested; the third band was polymorphic

with seven plants showing a higher and nine plants a lower molecular weight band. The same polymorphism was observed in the DH progeny with similar frequencies (ca. 1:1 ratio). This polymorphism was presumed to have preexisted in the parents and was not included in the overall summary of the methylation polymorphism.

Discussion

RLFP and RAPD techniques were employed to evaluate the level of gametoclonal variation induced in barley by AC and Hb systems of doubled haploid production. Both techniques are capable of detecting point mutations and larger rearrangements of DNA, although only the former allows discrimination between the two types of genetic alterations. Since the majority of restriction enzyme recognition sites (in RFLP) and oligonucleotide priming sites (in RAPD) are probably outside of the coding sequences, these techniques detect genetic variation not subjected to phenotypic selection. Snape et al. (1988) suggested that barley has lower levels of gametoclonal variation than wheat due to its diploid nature causing negative selection of deleterious mutations. Employing selection-neutral markers in comparative analyses of gametoclonal variation in these two species would facilitate critical evaluation of this hypothesis.

Point or deletion/insertion mutations were not detected in Hb or Ac-derived DHs by RFLP or RAPD even though a substantial length of DNA was analyzed. Subtelomeric heterochromatin and rDNA sequences, reported to be particularly sensitive to somaclonal variation (Larkin et al. 1984; Breiman et al. 1987; Rode et al. 1987; Karp et al. 1992), did not show any detectable changes. The apparently high genetic stability of barley AC-derived doubled haploids seems to be in contrast to the results of Ullrich et al. (1991) who reported an average of 17.2% and 7.8% somaclonal variation among immature embryo callus-derived R2 plant rows and R2 head rows, respectively. It is possible that changes in the methylation pattern detected with high frequency in the AC population may account for this discrepancy, since methylation may play an important role in gametoclonal variation (Brown 1989).

Molecular studies of gametoclonal variation in doubled haploids are limited to analyses of the plastid genome (Day and Ellis 1985) and repetitive sequences (De Paepe et al. 1983; Rode et al. 1987) of AC-derived plants. However mapping of the barley genome using DH populations provides numerous opportunities for observing non-parental RFLP bands. Putative mutations were not detected in an AC-derived population from the cross Steptoe × Morex (P. Devaux et al. in preparation). Mutations were also not reported for Igri × Franka or Proctor × Nudinka mapping with AC-derived populations (Graner et al. 1991; Heun et al. 1991). Extensive mapping of a Steptoe × Morex Hb-derived DH population (Kleinhofs et al. 1993) provided no evidence for mutational variation induced by this technique. Although mapping may not be influenced by rare, mutation-generated RFLP bands, we believe that if such bands had occurred in significant frequencies they would have been reported. These data indicate that AC- and Hb-derived DHs have very low levels of induced DNA changes in barley.

The level of DNA methylation was altered in AC and to a much smaller extent, in Hb-derived DHs. The most common methylated base, 5-methylcytosine, accounts for up to 30% of C in plant DNA (Gruenbaum et al. 1981; Adams and Burdon 1985) depending on the genome size (Adams 1990). Barley, with a genome size of 5.5×10^9 bp, has 25.7% of the C residues methylated (Amasino et al. 1990).

Brown et al. (1990) reported high levels of RFLPs in the R1 generation of protoplast-derived rice (*Oryza sativa* ssp *japonica*) plants both with methylation sensitive and insensitive restriction enzymes. Higher genetic instability of the actin genes was found in rice plants regenerated from calli maintained for 67 days versus 28 days in culture (Müller et al. 1990). In our study, 96% (49 out of 51) of the total methylation polymorphism was from AC-derived DHs. These changes in the DNA methylation pattern were probably induced during the in vitro culture of the microspores. The longer period in culture for the AC-versus the Hb-derived DHs might account for the higher level of C methylation polymorphism observed. The two variants observed in the Hb-derived DHs could be related to the fact that a few haploid embryos gave rise to calli prior to regeneration and, therefore, had to be maintained longer (up to 6 weeks) in vitro (Pickering and Devaux 1992).

Apart from the tissue culture conditions, AC and Hb systems start with different tissues: microspores versus immature zygotic embryos. Different tissues may have different levels of DNA methylation as reported for tomato (Messeguer et al. 1991). We cannot exclude the possibility that different methylation levels that could be heritable preexisted in the material used to initiate the DH development. However the relationships between different methylation patterns and their heritability are obscure.

Dramatic differences among the probes in the level of methylation pattern polymorphism detected indicates that DNA methylation changes do not occur at random throughout the genome. This is in agreement with the results of Brown (1989). The differences among the cDNA probes may be attributed to the differences in gene activity during the culture phase among the corresponding genes. It is unlikely that the observed changes in methylation pattern could have been induced by colchicine since a high proportion of spontaneous DHs are usually recovered from AC while no spontaneous doubling occurs in the Hb system (Pickering and Devaux 1992).

The heterogeneity detected by the probe 21B9 among the plants of cv Igri provides an example of hidden polymorphism present in barley cultivars. Kilian and Gale (1991) reported similar RFLP polymorphism in the population derived from cv Triumph. Polymorphism for the nicotinamide adenine dinucleotide (NADH) nitrate

reductase gene in cv Steptoe was observed in our laboratory and shown to be due to the presence of different alleles in the parent lines (Jeter 1987). It was not possible to verify the source of Igri polymorphism since the parents were not available. However the 1:1 ratio observed for the two bands suggests that these bands are inherited from parental genotypes.

An interesting aspect of cv Igri polymorphism comes from the fact that it was detected with a methylation-sensitive enzyme *Hpa*II, while being absent for three methylation-insensitive enzymes. Since *Hpa*II is a "4-cutter" and both alternative bands are several kb in size, we may assume that there is a methylation pattern polymorphism rather then differences in the distribution of the recognition sequence. If our assumptions about the source and nature of this polymorphism are correct, it may represent an example of high stability of methylation pattern polymorphism. Interestingly the same probe (21B9) detected very high levels of methylation pattern polymorphism in AC-derived DHs. The fact that nearly half of the tested probes detected very high levels of polymorphism among AC-derived DHs together with proven stability of methylation changes (Brown 1989; Brettell et al. 1991) and their Mendelian inheritance (Messeguer et al. 1991) suggests the possibility of using this system for generating novel RFLPs. This could find numerous applications both in theoretical and applied RFLP research in highly non-polymorphic populations.

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