Theor Appl Genet (1991) 83:71–80

THEORETICAL AND APPLIED GENETICS

Comprehensive molecular characterization of tissue-culture-derived *Hordeum marinum* plants

D. Shimron-Abarbanell and A. Breiman*

Department of Botany, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Received January 16, 1991; Accepted April 18, 1991
Communicated by K. Tsunewaki

Summary. Scuttelar calli of *Hordeum marinum* readily and efficiently regenerate functional plants. In order to assess genetic variability among the regenerants we employed multiple analytic tools, which included molecular and biochemical assays. Total DNA extract from regenerated plants was digested with at least two restriction enzymes and hybridized to four nuclear and six mitochondrial coding sequences, in addition to one nuclear and three mitochondrial noncoding probes. SDS-PAGE analyses of hordein extracted from seeds of regenerated plants and activity assays of α-amylase were also performed. The nuclear and mitochondrial genomes of 50 regenerated plants demonstrated relative stability when assessed with coding sequences and by biochemical analyses. However, the mitochondrial noncoding probes revealed one qualitative somaclonal variant characterized by a loss of a hybridizing fragment. Moreover, changes in the methylation patterns of the rRNA genes and the nontranscribed spacer were revealed in another regenerated plant. The albino plant regenerated was characterized by a loss of three chloroplast DNA *Bam*HI fragments.

Key words: *Hordeum marinum* – Regenerated plants – Nuclear genome – Mitochondrial genome – Somaclonal variation

Introduction

Plant regeneration from cultured tissues is an asexual process which could be expected to give rise to clonal uniformity. Plant tissue culture instability has been documented in some plant species. There are contradictory reports about somaclonal variation in cereals, ranging from considerable variation in wheat (Larkin et al. 1984) and rice (Sun et al. 1983), to little or no variation in barley (Breiman et al. 1987b; Karp et al. 1987), *Pennisetum* (Swedlund and Vasil 1985) and *Panicum* (Hanna et al. 1984).

Molecular studies on somaclonal variation in wheat and barley (Breiman et al. 1987a; Rode et al. 1987a) have demonstrated that the NOR loci (nuclear organizer region) coding for rRNAs and the electrophoretic pattern of storage proteins of wheat and barley are very sensitive parameters for assessing somaclonal variation (Larkin et al. 1984; Breiman et al. 1987b; Karp et al. 1987). Changes in methylation of the rRNA genes were also documented in soybean suspension cultures (Quemada et al. 1987). However, no variations were observed when wheat-regenerated plants and progenies were studied for their rDNA methylation patterns (Rode et al. 1987a).

Evidence of a direct relationship between mitochondrial genome stability and regeneration capacity was reported by Rode and coworkers (1988). Whereas wheat embryogenic calli showed mtDNA organization identical to that of the parental plants, variation was observed in nonembryogenic calli.

The involvement of the chloroplast genome in the appearance of albino plants was studied both with electron microscopy (TEM) and with recombinant DNA techniques (Day and Ellis 1984, 1985; Chen et al. 1988). Hybridizations of chloroplast DNA derived from albino plantlets to cloned DNA revealed that a large part of the chloroplast genome, including the inverted repeats, was missing in wheat and barley albino plantlets (Day and Ellis 1984, 1985).

In the present study, a comprehensive analysis of the organization of the coding sequences of the nuclear genome, the noncoding sequences of the nuclear genome, the coding regions of the mitochondrial genome, and the

* To whom correspondence should be addressed

noncoding regions of the mitochondrial genome was performed.

In a previous work (Rotem-Abarbanell and Breiman 1989) regeneration of *H. marinum* plants was reported. A high regeneration capacity (94%) from immature-embryo-derived calli was observed.

In the present study, we report the relative stability of the nuclear and mitochondrial genomes of *H. marinum*-regenerated plants. In addition, we describe qualitative changes of the chloroplast genome of a regenerated albino plant.

Materials and methods

Plant material

Hordeum marinum plants were regenerated from the scutellar calli of immature and mature embryos (Rotem-Abarbanell and Breiman 1989). A total of 50 regenerated plants (R_0) derived from 14 different explants (3 from mature embryos and 11 from immature embryos) was chosen for molecular analyses.

DNA extraction, endonuclease digestion, and Southern blot hybridization

DNA was extracted according to Dvorak and Appels (1982) with minor modifications as described previously (Breiman et al. 1987b). Briefly, 1 g of green leaves was ground to powder with liquid nitrogen in a mortar and pestle. The powder was incubated for 1 h at 37 °C with 500 µl S buffer (100 m*M* TRIS-HCl (pH 8.8), 100 m*M* NaCl, 50 m*M* EDTA, 2% SDS) containing 0.1 mg/ml proteinase K. After centrifugation, the supernatant was extracted once with phenol-chloroform (1:1 v/v) and once with chloroform: isoamyl alcohol (24:1 v/v) and DNA were precipitated from the aqueous phase with 2.5 vol. of cold ethanol. The precipitate was dried and resuspended in 300 µl TE buffer (10 m*M* TRIS-HCl (pH 8.0), 1 m*M* EDTA), and incubated for 30 min at 37 °C with 60 units/ml RNase T1 (Sigma) and 100 µg/ml RNase A (Sigma). Re-extractions with phenol-chloroform and chloroform and ethanol precipitation were performed as above. The DNA pellet was dried and redissolved in 50 µl TE buffer.

The DNA was digested with the following restriction enzymes: *Bam*HI, *Hae*III, *Hind*III, *Rsa*I, *Sac*I, and *Taq*I. For rDNA methylation analyses, double digestion was used: *Bam*HI + *Msp*I and *Bam*HI + *Hpa*II.

The digested DNA was separated on agarose gels and transferred to nylon filter (Gene Screen Plus, NEN Research Products) according to Southern (1975). The filters were baked for 2 h at 80 °C and then prehybridized for 6 h in a plastic bag according to the manufacturer's instructions. For hybridization a ^{32}P-labelled probe and 100 µg/ml sheared salmon sperm DNA were injected into the plastic bag and incubated for at least 16 h. The probes were removed according to the manufacturer's instructions (Gene Screen Plus, NEN Research Products) for the reusage of the membranes.

Construction of mtDNA shotgun library and identification of noncoding clones

Barley mitochondrial DNA and pUC18 DNA were digested to completion with *Bam*HI. The digested mtDNA was ligated to the dephosphorylated vector with T_4 ligase (Boehringer-Mannheim) according to Maniatis et al. (1982). For the identification of colonies containing noncoding sequences of mtDNA, 50 random clones were chosen from the library, and their DNA was extracted (Maniatis et al. 1982) and digested with *Bam*HI. The digested DNA was separated on 1% agarose gel, blotted to nylon filters (Gene Screen Plus, NEN Research Products), and hybridized to known coding sequences. Three clones that exhibited hybridization to any of the coding sequences were chosen for the assessment of mitochondrial noncoding sequences (Table 1).

Recombinant DNA clones

Table 1 provides the list of plasmids that were used for the assessment of the nuclear, mitochondrial, and chloroplast DNA organization and specifies the respective vectors, cloning sites, fragment sizes, and their origin.

Hordein and α-amylase assays

Hordein extraction and separation by SDS-PAGE were performed according to Shewry et al. (1978) with minor modifications (Breiman et al. 1987b).

Five seeds of *H. marinum* from each regenerated plant were screened for the production of the aleurone secretory enzyme α-amylase, according to Ho et al. (1980). The seeds were surface sterilized with sodium hypochloride and the endosperm halves were assayed for GA_3 induction and ABA repression of α-amylase synthesis. The assay medium contained 0.15% (w/v) commercial potato starch, 40 m*M* $CaCl_2$, 20 m*M* sodium succinate, 10 µg/ml chloramphenicol, and 1% agar. For GA_3 induction assay, 1 µ*M* GA_3 was added to the medium. GA_3 (0.5 µ*M*) and ABA (100 µ*M*) were added for the ABA inhibition tests. The endosperm halves were incubated for 48 h at 25 °C in the dark. The seeds were removed and the surface was washed with a solution of 36 m*M* KI and 2.4 m*M* I_2 in 0.05 *N* HCl. The white haloes were measured and compared to parental controls. The results were analyzed according to Larkin et al. (1984). Each assay was performed twice on three seeds that were randomly chosen from each regenerated plant.

Results

Analysis of ribosomal DNA spacer length polymorphism

DNA was extracted from each of the 50 plants regenerated from the scutellar calli derived from immature and mature embryos. The DNA was digested with two restriction enzymes, separated on agarose gels, blotted, and hybridized with ^{32}P-labeled pHv294 (Table 1).

*Taq*I endonuclease was chosen because it cleaves the barley rRNA genes to short fragments, whereas the non-transcribed intergenic spacer produces long fragments (Gerlach and Bedbrook 1979). *Sac*I endonuclease cleaves the intergenic spacer and the ribosomal coding genes into variable and fixed length fragments, respectively (Saghai-Maroof et al. 1984). The spacer length in the *Taq*I digestion revealed two spacer fragments of 0.92 kb and 0.83 kb (Fig. 1a) and seven fragments of the ribosomal genes with lengths shorter than 0.72 kb. The two fragments of the *Sac*I digestion corresponding to the spacer length were 5.2 kb and 4.6 kb, whereas the rRNA gene fragment was 3.6 kb long (Fig. 1b). No variation in fragment number, size, or relative intensity of hybridization

Table 1. Clones used for the assessment of the nuclear, mitochondrial, and chloroplast genome organization of *Hordeum marinum*-regenerated plants

Probe	Plasmid designation	Content of plasmid	Vector	Cloning site	Size of inserted fragment (kbp)	Origin of sequence	Reference
Nuclear	pHv 294	18S, 26S, and non-transcribed spacer or rDNA	pAC184	*Eco*RI	9	*H. vulgare*	Gerlach and Bedbrook 1979
	pHv 294/4	18S and 26S rDNA	pUC18	*Bam*H1	4	*H. vulgare*	Subclone of the pHv 294
	pHv 294/2	Nontranscribed spacer of rDNA	pUC18	*Bam*H1	2.3	*H. vulgare*	Subclone of the pHv 294
	pB 11	cDNA of B hordein	pUC18	*Hin*dIII	0.844	*H. vulgare*	Forde et al. 1981
	pCP 387	cDNA of C hordein	pUC18	*Hin*dIII	0.475	*H. vulgare*	Forde et al. 1985
	2128	α-amylase	pBR322	*Pst*I	1.5	*H. vulgare*	Baulcombe and Buffard 1983
Mito-chondrial	*atp9*	Subunit 9 of ATPase and noncoding sequences at the 5′ and 3′ flanking regions	pUC19	*Xba*I	2.2	*Zea mays*	Dewey et al. 1985b
	atp6	Subunit 6 of ATPase and noncoding sequences at the 5′ and 3′ flanking regions	pUC13	*Hin*dIII	2.7	*Zea mays*	Dewey et al. 1985a
	atpα	Subunit α of ATPase and non-coding sequencesat the 5′ and 3′ flanking regions	pUC19	*Hin*dIII	4.2	*Zea mays*	Braun and Levings 1985
	M8a (*5′cob*)	5′ region of the apocytochrome b	M13	*Sau*3A *Hin*dIII	0.250 0.250	*Triticum aestivum*	Boer et al. 1985
	L4C (*5′coxI*)	5′ region of the gene coding for cytochrome c oxydate subunit I and a noncoding sequence at the 5′ flanking region	M13	*Hin*dIII *Pst*I	0.900	*Triticum aestivum*	L. Bonen (personal communication)
	T11 (*5′coxII*)	5′ region of the gene coding for cytochrome c oxydate subunit II	M13	*Taq*I	0.230	*Triticum aestivum*	Bonen et al. 1984
	pBmt9	Noncoding sequence of mitochondrial DNA	pUC18	*Bam*H1	6.6	*H. vulgare*	(see 'Mat. and Meth.')
	pBmt11	Noncoding sequence of mitochondrial DNA	pUC18	*Bam*H1	0.590	*H. vulgare*	(see 'Mat. and Meth.')
	pBmt30	Noncoding sequence of mitochondrial DNA	pUC18	*Bam*H1	1.0	*H. vulgare*	(see 'Mat. and Meth.')
Chloro-plast	pHvc P1 pHvc P3–10	Chloroplast sequences	pAT153	*Pst*I	P1-20.7 P3-18.9 P4-13.4 P5-11.9 P6-10.7 P7- 9.9 P8- 8.1 P9- 5.1 P10-5.2	*H. vulgare*	Day and Ellis 1985

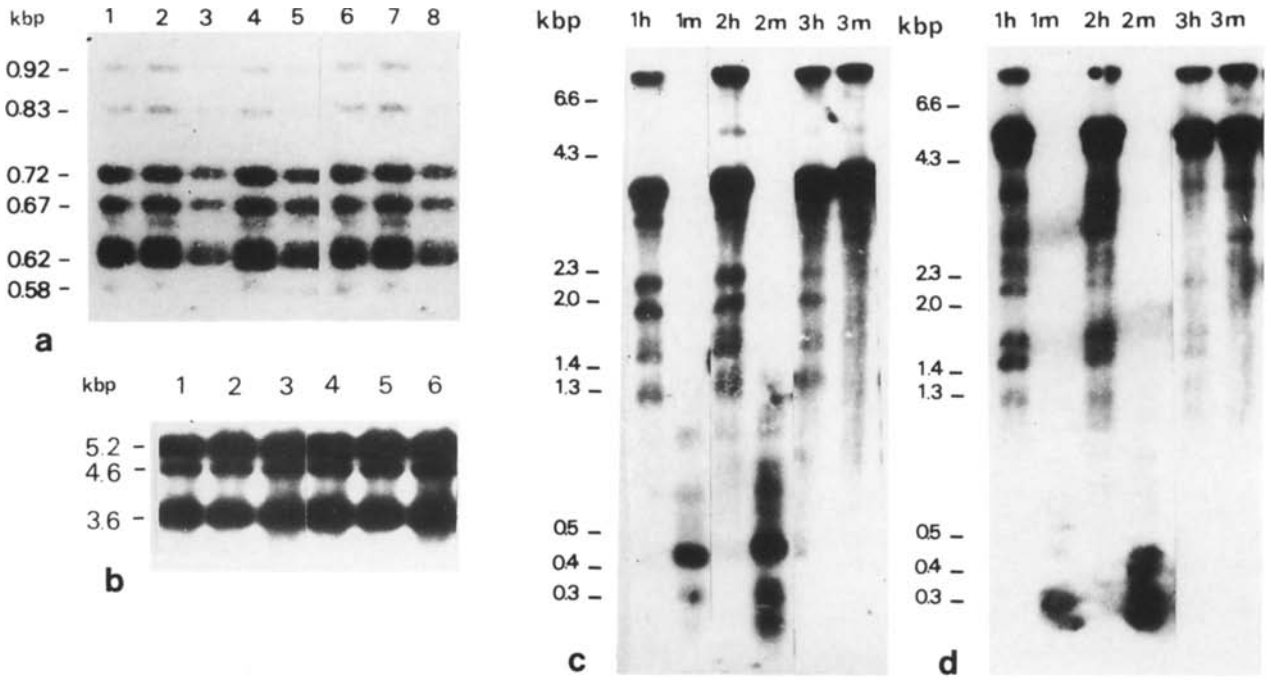

Fig. 1 a–d. Southern blot hybridization analyses of total genomic DNA of *Hordeum marinum*-regenerated plants with rDNA probes. Blots of *Taq*I (**a**) and *Sac*I (**b**) digests hybridized with the pHv294 probe consisting of both the rDNA genes and nontranscribed spacer. Methylation patterns of the rDNA genes (**c**) and nontranscribed spacer (**d**) were analyzed by double digest of *Bam*H1 + *Hpa*II (*h*) and *Bam*H1 + *Msp*I (*m*). Lane *1* = control; lanes *2* and *3* = regenerated plants

was observed among the regenerated plants (Fig. 1 a). Only 7 and 5 out of 50 regenerated plants in addition to one parental plant are represented in Fig. 1 a and b, respectively.

Methylation patterns of the nontranscribed intergenic spacer and the genes coding for the rRNA

Further assessments of the rRNA genes and intergenic spacer sequences were carried out by analyzing the methylation patterns of the rDNA of 20 regenerated plants. Consequently, samples of DNA were first cleaved with *Msp*I or *Hpa*II and then digested wtih *Bam*HI. The isochisomers *Msp*I and *Hpa*II recognize the sequence CCGG. However, *Hpa*II can cleave the sequence when the 5′ deoxycytosine is methylated, whereas *Msp*I digests the sequence C^mCGG. The second digest with *Bam*HI was chosen as this enzyme separates the ribosomal genes and the nontranscribed spacer into two distinct fragments (Gerlach and Bedbrook 1979).

The cloned (nonmethylated) sequences of the rRNA genes (pHv294/4) and the nontranscribed spacer (-NTS-) (pHv294/2) produce five and three DNA fragments, respectively, when digested with *Msp*I or *Hpa*II (data not shown). Since the diploid genome of barley contains 10^4 copies of rDNA, one could expect various degrees of methylation in the 5′ and 3′ deoxycytosine of the CCGG sites present among these numerous copies. Indeed, the partial hybridization patterns of the rRNA genes and NTS revealed by the *Msp*I and *Hpa*II digests demonstrate various degrees of methylation of both deoxycytosines (Fig. 1 c, d). However, since the *Hpa*II digest revealed a more complex hybridization pattern than the *Msp*I (Fig. 1 c, d, lanes 1 h and 1 m), we concluded the 3′ deoxycytosine was more methylated than the 5′ deoxycytosine. It should be noted that the two smallest fragments produced by digestion of cloned rRNA genes (pHv294/4) with *Msp*I were not revealed in the hybridization patterns of total DNA. This may be due to poor transfer of small fragments to the nylon membrane.

The DNA from 19 out of 20 tested plants exhibited identical methylation patterns of both the rRNA genes an the intergenic spacer. The methylation patterns of only two regenerated (Fig. 1 c, d, lanes 2, 3) an one parental plants are represented in Fig. 1 c, d. The somaclonal variant (Fig. 1 c, d, lanes 3 h and 3 m) exhibited a higher degree of methylation of both deoxycytosines of the CCGG sequence.

Electrophoretic pattern of storage protein by SDS-PAGE and organization of the B and C hordein coding sequences

To detect culture-induced changes in the electrophoretic profiles of hordeins, we extracted these proteins from five seeds of each individual regenerated plant and loaded the extracts on SDS-PAGE. Extracts from all 50 plants pro-

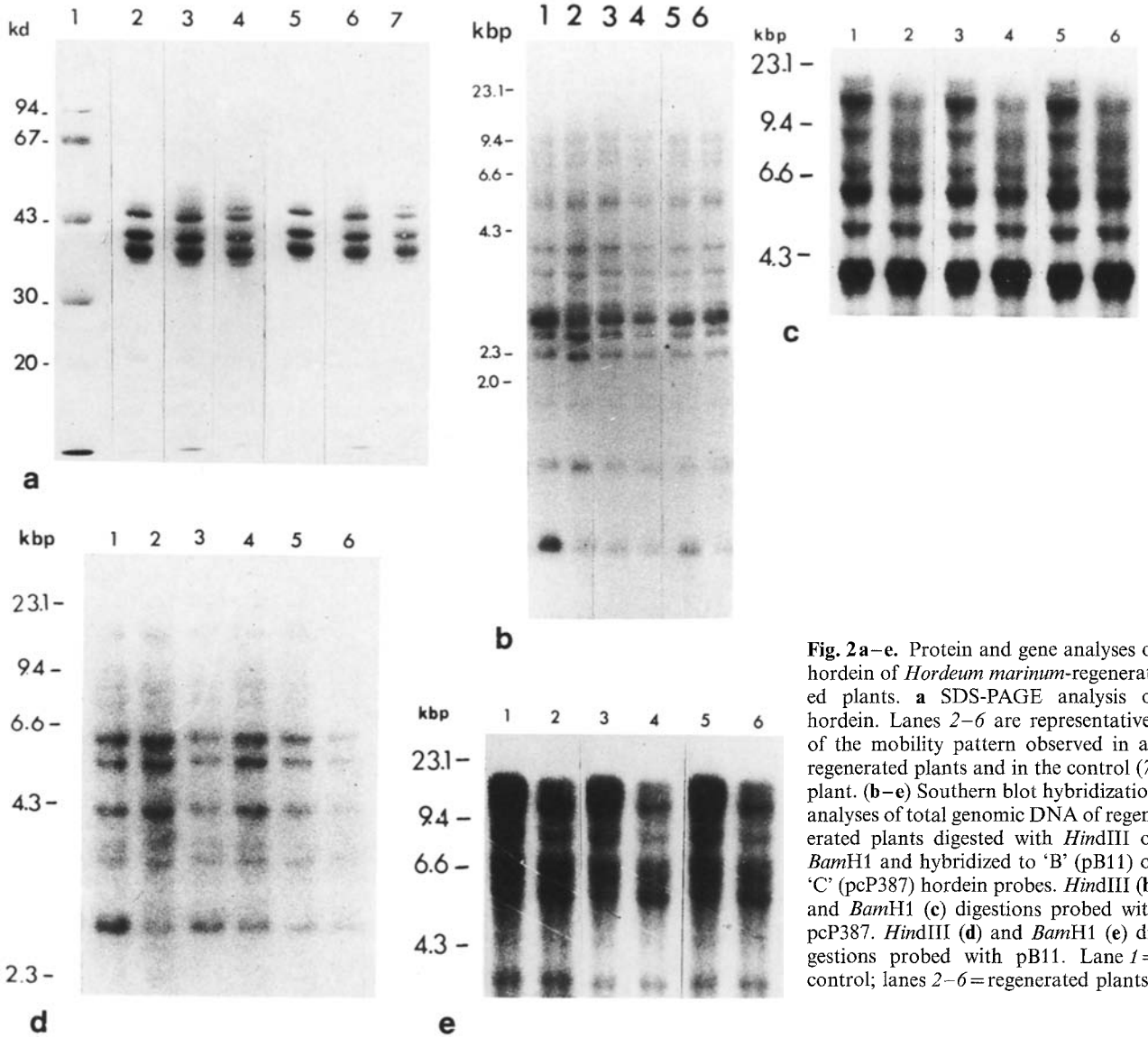

Fig. 2a–e. Protein and gene analyses of hordein of *Hordeum marinum*-regenerated plants. **a** SDS-PAGE analysis of hordein. Lanes *2–6* are representatives of the mobility pattern observed in all regenerated plants and in the control (*7*) plant. (**b–e**) Southern blot hybridization analyses of total genomic DNA of regenerated plants digested with *Hin*dIII or *Bam*H1 and hybridized to 'B' (pB11) or 'C' (pcP387) hordein probes. *Hin*dIII (**b**) and *Bam*H1 (**c**) digestions probed with pcP387. *Hin*dIII (**d**) and *Bam*H1 (**e**) digestions probed with pB11. Lane *1* = control; lanes *2–6* = regenerated plants

vided the same profile. The profiles of six samples are presented in Fig. 2a.

Since the polymorphism that may be detected in the organization of these genes is not necessarily expressed at the protein level, we also analyzed the organization of the B and C hordein coding sequences. Uniformity in the hybridization patterns of the DNA of all regenerated plants to the B and C hordein probes was observed. The hybridization patterns of 5 out of 50 regenerated plants in addition to one parental plant are represented in Fig. 2b–e.

α-Amylase activity and gene organization

In vitro culture may cause changes in the structural genes that encode enzymes or in the controlling elements that regulate the expression of such genes. We chose the α-amylase because it was amply investigated in barley and its induction is strongly regulated by two growth substances: GA_3 and ABA. To identify somaclonal variants that lost their sensitivity of α-amylase induction by GA_3 and repression by ABA, we used the procedure of Larkin et al. (1984). According to this procedure, no variants were detected among the R_1 seeds of *H. marinum*. Seven out of 50 regenerants tested and one parental plant are represented in Fig. 3a.

Although no variation in α-amylase activity among regenerated plants could be observed, the organization of the α-amylase coding sequences was analyzed by Southern blots. The DNA extracted from green leaves was digested with *Bam*HI or *Hin*dIII and hybridized to a cDNA clone of α-amylase (Table 1).

In the *Hin*dIII digestion (Fig. 3b), three major fragments (16.0, 11.9, and 9.2 kb) hybridized to the α-amy-

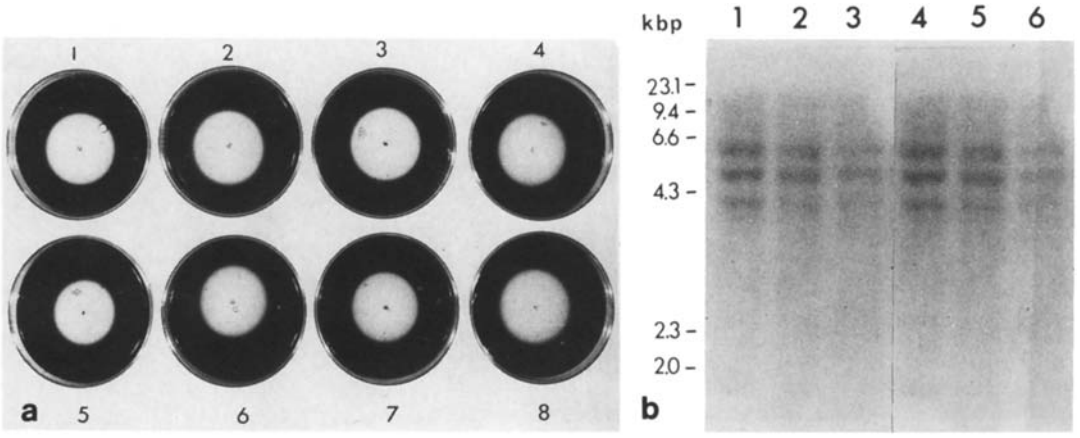

Fig. 3a and b. **a** α-amylase activity induced by GA_3 in seeds of regenerated *Hordeum marinum* plants. Lane *1* = control; lanes *2–8* = regenerated plants. **b** Southern blot hybridization analysis of total genomic DNA of regenerated plants, digested with *Hin*dIII hybridized with an α-amylase probe. Lane *1* = control; other lanes contain samples from regenerated plants

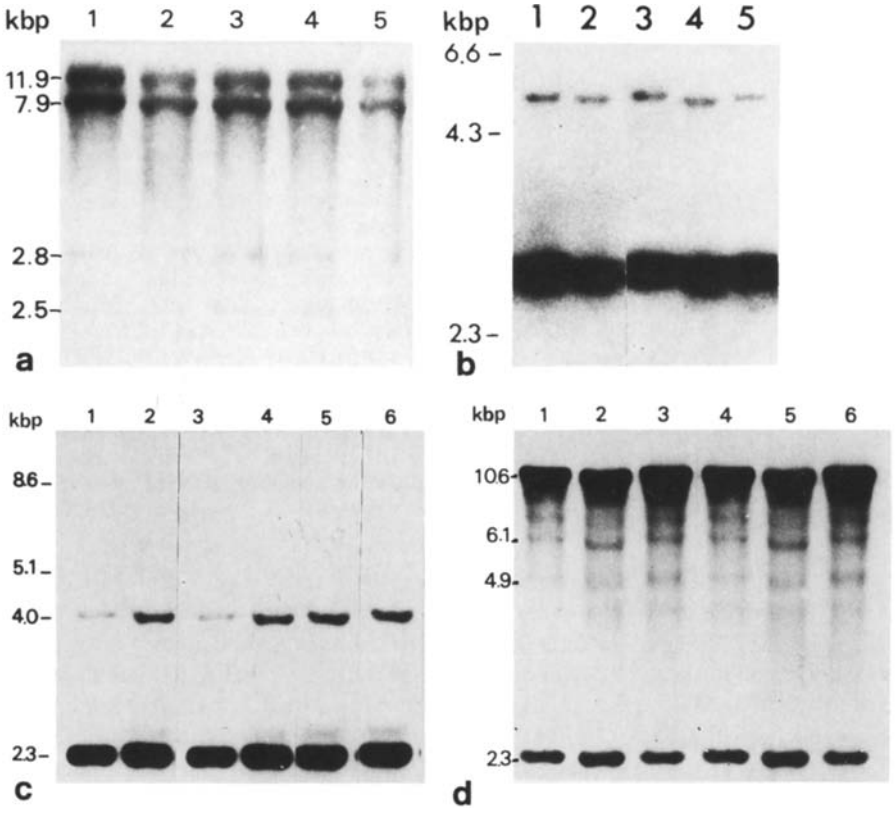

Fig. 4a–d. Southern blot hybridization analyses of *Hin*dIII (**a–c**) or *Bam*HI (**d**) digested genomic DNA with mitochondrial probes containing the coding sequences: *atp 9* (**a**) and *atp 6* (**b–d**). Lane *1* = control; other lanes represent regenerated plants

lase probe. The α-amylase hybridization pattern was identical in all the R_0 and control plants, and 3–5 of 50 hybridization patterns of regenerated plants in addition to one parental plant are represented in Fig. 3b.

Mitochondrial DNA organization

The mitochondrial coding sequences among cereals are highly conserved (Bonen et al. 1984; Boer et al. 1985). Therefore, we have used several wheat and maize mitochondrial genes as heterologous probes to characterize the organization of the mtDNA sequences on Southern blots of total DNA. This allowed the screening of the regenerated *H. marinum* plants without the need to purify mitochondrial DNA.

Total DNA was digested with *Hin*dIII or *Bam*HI and hybridized with several ^{32}P-labelled DNA clones of mi-

Table 2. Southern hybridization of total DNA of *H. marinum*-regenerated plants with mitochondrial probes

Probe	Endonuclease	No. of restriction fragments hybridized to the probe	Length of hybridization fragments (kbp)
5′ *cox*I	*Hin*dIII	5	6.6, 5.5, 3.2, 2.1, 2.0[a]
	*Bam*H1	6	7.0, 5.2, 3.9, 2.6, 2.4, 2.1
5′ *cox*II	*Hin*dIII	5	11.9, 7.9, 2.9, 2.5, 1.9
	*Bam*H1	1	1.42
5′ *cob*	*Hin*dIII	2	2.9, 2.8
	*Bam*H1	1	4.0
atpα	*Hin*dIII	3	5.2, 2.8, 2.7
	*Bam*H1	3	5.1, 3.5, 3.2
atp6	*Hin*dIII	5	8.6, 5.1, 4.0, 2.5, 2.3
	*Bam*H1	6	10.6, 6.7, 6.1 4.9, 4.3, 2.3
atp9	*Hin*dIII	3	5.2, 2.8, 2.7
	*Bam*H1	3	5.1, 3.5, 3.2
pBmt9	*Hae*III	4	2.21, 1.68, 1.65, 1.56
	*Rsa*I	15	1.92, 1.73, 1.63, 1.49, 1.29, 1.20, 1.14, 1.05, 0.98, 0.89, 0.78, 0.73, 0.68, 0.62, 0.59
	*Taq*I	6	1.79, 1.58, 1.34, 1.32, 1.30, 1.25
pBmt11	*Hae*III	2	1.84, 1.77
	*Rsa*I	7	1.54, 1.46, 1.27, 0.95, 0.81, 0.71, 0.67
	*Taq*I	5	1.75, 1.39, 1.29, 1.26, 1.19
pBmt30	*Hae*III	1	1.73
	*Rsa*I	6	1.2, 1.54, 0.93, 0.87, 0.66, 0.58
	*Taq*I	3	1.80, 1.50, 1.30

[a] A very weak hybridization signal observed in total DNA extracts
__ DNA fragment which hybridized strongly to the probe. The hybridization signals of the other DNA fragments were less intense

tochondrial genes (Table 2). Hybridization of the *Hin*dIII-digested DNA with the 5′*coxI* probe revealed four fragments ranging from 6.6 to 2.1 kbp (Table 2; Fig. 4a, lanes 1–3). *Bam*HI-digested DNA exhibited six fragments with homology to the same probe (Table 2). The multiplicity of the bands obtained in *H. marinum* may be explained by the occurrence of the gene in more than one site in the genome (Lonsdale 1989), or the presence of *Hin*dIII and *Bam*HI sites within the gene. The latter possibility is less probable, and the coding region of the *coxI* gene is conserved between maize and wheat and does not contain *Hin*dIII or *Bam*HI sites (Bonen et al. 1984). However, since the *coxI* probe used for the analyses (Table 1) contains a noncoding sequence flanking at the 5′ region of the gene, the multiplicity of bands observed may be due to the presence of *Hin*dIII and *Bam*HI sites in this region. Hybridization of the Southern blots with the coding sequences revealed an identical pattern among the DNAs extracted from all the analyzed regenerated plants (Fig. 4a–d; Table 2). The similar pattern of hybridization observed for the *atpα* and *atp9* suggests that these genes are adjacently located on the same *Hin*dIII and *Bam*HI fragments (Begu et al. 1989).

Southern blots of total DNA extracted from the regenerated plants of *H. marinum*, digested with *Bam*HI and *Hin*dIII, and hybridized with six mitochondrial coding sequences revealed a total of 42 identical fragments (Table 2). In Fig. 4a–d only a sample of 4 or 5 out of 50 regenerated plants are represented.

An additional approach was taken to assess the stability of the mitochondrial genome of the regenerated plants. DNA from 40 different plants was digested with three endonucleases, each having a recognition site of 4 bp (*Taq*I, *Rsa*I, and *Hae*III). In addition, three barley mitochondrial noncoding sequences were used as probes (Table 1). Although the *Hae*III digests produced a small number of hybridizing fragments with these probes (Table 2), as many as 15 hybridizing bands were observed when the DNA was digested with *Rsa*I and hybridized to the pBmt9 (Table 5c, Table 2). In one of the nine enzyme/probe combinations, a qualitative somaclonal variant was observed among the *H. marinum*-regenerated plants (Fig. 5b, lane 1). Quantitative variations were detected as well (Fig. 5b, lane 1; Fig. 5c, lanes 1 and 2). However, it should be noted that the observed quantitative variations may arise due to the presence or absence of mitochondrial subgenomic circles in the total DNA extracts used for the analyses. In Fig. 5a–c the hybridization patterns of 3 or 7 out o 40 regenerants, in addition to one parental plant, are demonstrated.

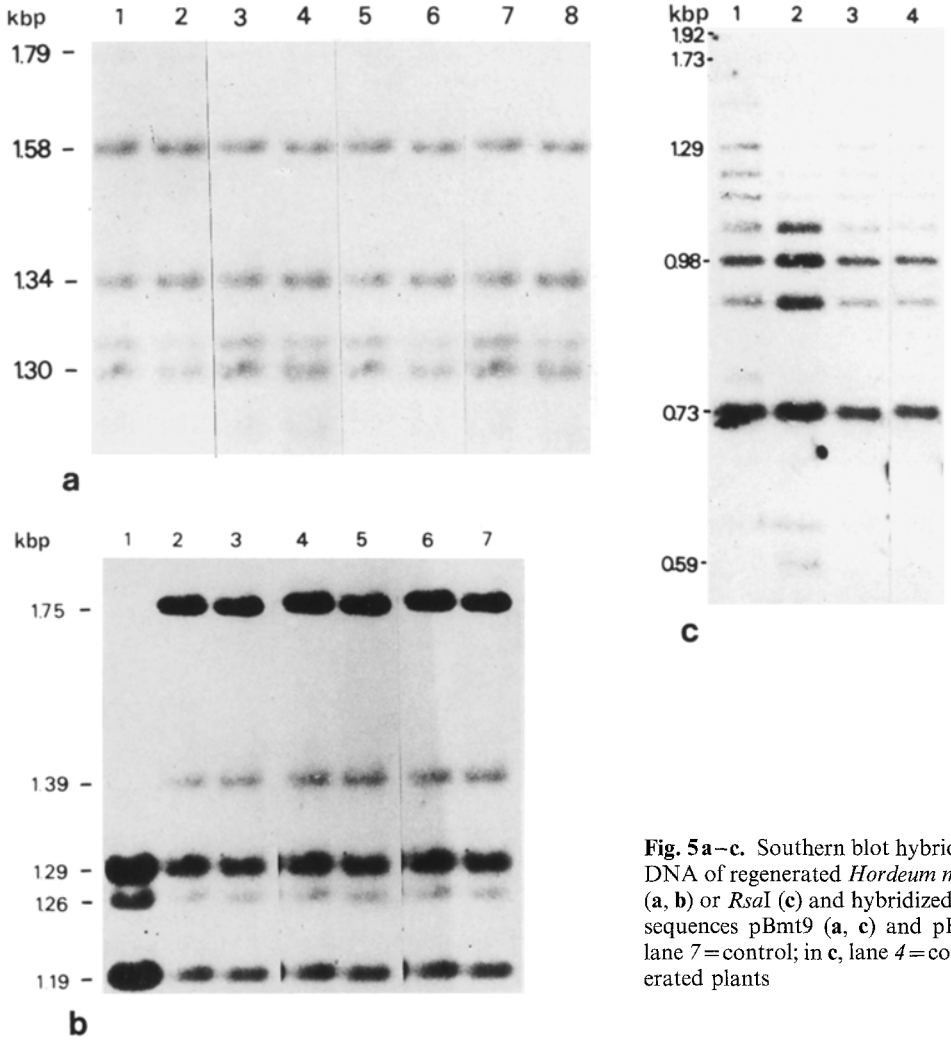

Fig. 5a–c. Southern blot hybridization analyses of total genomic DNA of regenerated *Hordeum marinum* plants digested with *Taq*I (**a**, **b**) or *Rsa*I (**c**) and hybridized to the mitochondrial non coding sequences pBmt9 (**a**, **c**) and pBmt11 (**b**), lane *8* = control; in **b**, lane *7* = control; in **c**, lane *4* = control; other lanes represent regenerated plants

Organization of albino chloroplast genome

The occurrence of albino plantlets among regenerants may indicate rearrangements or deletions in the chloroplast genome of these plants (Day and Ellis 1984, 1985). Total DNA from albino and green plants was digested with *Bam*HI and hybridized to nine probes derived from barley chloroplast DNA (Table 1; Fig. 6). Although identical amounts of green and albino DNA were used (10 µg), the hybridization intensities revealed by the albino plantlet's DNA were lower. A single variant of chloroplast DNA organization was detected in which three hybridization bands (8.2, 7.3, and 2.5 kb) were missing (Fig. 5 P_3), and were not revealed by longer exposures of the autoradiogram.

Discussion

This study demonstrates a relatively high genetic stability among the 50 regenerated *H. marinum* plants tested. Only two variants were observed: one in the methylation pattern of the rDNA and the other in the organization of a mitochondrial noncoding sequence.

Qualitative and quantitative differences were detected in the nontranscribed spacer of the rDNA of parental and regenerated wheat plants when their DNA was digested with *Eco*RI and *Msp*I (Rode et al. 1987a; Aubry et al. 1989). However, *Eco*RI + *Hpa*II digests of their DNA revealed identical hybridization patterns with the nontranscribed spacer. One *H. marinum* somaclonal variant exhibited a higher degree of resistance to both *Msp*I and *Hpa*II digestions than the parental plant. The differences were observed both in the nontranscribed spacer and in the rRNA genes.

Tissue culture manipulations may induce qualitative and quantitative variations in mitochondrial DNA of calli and cell suspensions (Rode et al. 1987b; Shirzadegan et al. 1989). In this study, we demonstrate changes in the mtDNA of a *H. marinum*-regenerated plant, characterized by loss of one hybridization band. Increase or de-

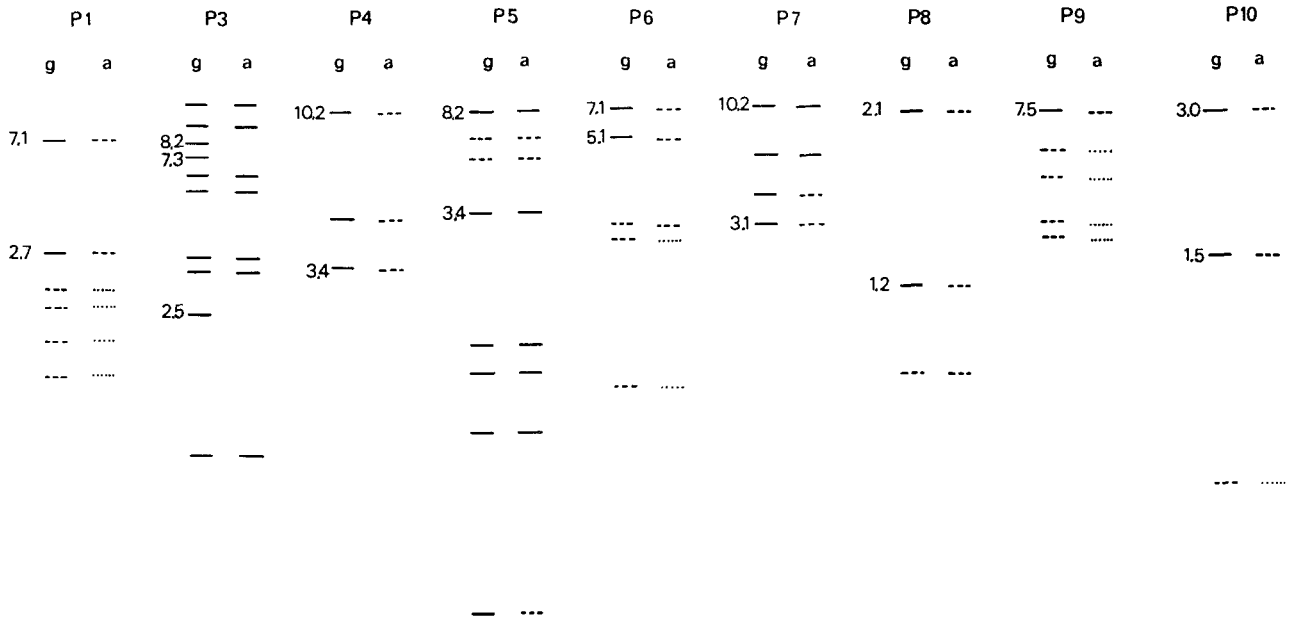

Fig. 6. Schematic representation of Southern blot hybridization analyses of total genomic DNA of a normal *Hordeum marinum* plant (*g*) and a regenerated albino plantelet (*a*) digested with *Bam*H1 and hybridized with chloroplast probes $pHvcP_1$–$pHvcP_{10}$ (P_1–P_{10}) (*dashed lines* indicate faint hybridization signals). Size indicator in kbp

crease in the relative stoichiometry of several hybridization fragments was also revealed. However, since differences in the hybridization intensity of total DNA and mtDNA with mitochondrial probes were revealed in preliminary experiments (data not shown), it may be assumed that the qualitative variations observed in the total DNA of the regenerated plants are not solely attributed to somaclonal variation.

The alterations occurred in noncoding sequences of the mitochondrial genome (Fig. 5b). However, coding sequences of the mtDNA remained stable through tissue culture manipulations (Fig. 4b–e). This may suggest a selection mechanism that restricts the regeneration of plants to cells having an unaltered mitochondrial coding capacity. Recent work of Rode et al. (1988) provided evidence for direct relation between regeneration ability of callus culture and mitochondrial DNA organization. An 8-kb mtDNA fragment present in embryogenic calli was reduced or lost in nonembryogenic ones.

The occurrence of albino plantlets derived from *in vitro* manipulations may be due to alterations of the chloroplast genome (Day and Ellis 1984, 1985). Our studies demonstrated that although most of the albino plantlet's chloroplast genome tested was unaltered qualitatively, the hybridization signals were in most cases less intense than those observed in the green plant (Fig. 6).

The results presented in this study indicate that barley exhibit relative stability through *in vitro* manipulations. Several factors may affect this stability. The ploidy level may play an important role in producing somaclonal variation. Whereas diploid species of genera such as *Hordeum*, *Secale*, and *Panicum* tend to exhibit genetic stability, polyploid species of the Gramineae genera (e.g., *Triticum*, *Avena*) were reported to produce *in vitro*-derived regenerants with a high frequency of variation (McCoy et al. 1982; Larkin et al. 1984; Linacero and Vazquez 1986). The somaclonal variation revealed in the regenerated (diploid) maize plants may be attributed to the activity of transposable elements (Peschke et al. 1987). Regeneration mode may affect the genetic stability of plants as well (Morrish et al. 1987; Rode et al. 1988). Somaclonal variation was observed in high frequencies in plants originating from nonembryogenic cultures through organogenesis, whereas plants derived from embryogenic cultures through embryogenesis exhibit genetic stability. Therefore, the relative genetic stability of the *H. marinum*-regenerated plants may be attributed to their ploidy (2nd) and their origin (immature-embryo-derive calli).

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