Theor Appl Genet (1993) 85:926–930

THEORETICAL AND APPLIED GENETICS

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RFLP analysis of highly polymorphic loci in barley

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Received June 25, 1992; Accepted July 8, 1992
Communicated by F. Salamini

Summary. Barley middle-repeat sequences were screened for their ability to discriminate 51 barley commercial varieties. Two hordein clones, a clone encoding a leaf-specific thionin, a desiccation induced cDNA clone, a clone coding for 5S-rRNA and one corresponding to ubiquitin genes were tested. A very sensitive RFLP technique including four cutter restriction enzymes and denaturing 4% polyacrylamide gels were used to evidence the highest level of polymorphism.

The RFLP data were analyzed by computer. Some probe/enzyme combinations were able to differentiate a large number of the cultivars tested, whereas three probe/enzyme combinations succeeded in identifying all the varieties. The use of this RFLP method can thus be suggested for cultivar identification in barley.

Key words: RFLP analysis – Barley – B- and C-hordeins – Polymorphic loci

Introduction

Molecular probes revealing DNA restriction fragment length polymorphisms (RFLP) are being used as a tools to characterize the genetic variability of crop species (Beckmann and Soller 1983). Highly polymorphic RFLP markers are in this respect of particular use: in potato, the RFLP marker GP35 has revealed 122 unique restriction patterns in the 134 varieties evaluated (Görg et al. 1992). Yet for several self-pollinated species the level of DNA polymorphism appears to be relatively low (Gebhardt and Salamini 1992). In barley, for instance, highly polymorphic probes that would be useful for investigating genetic variability in a large number of cultivars have yet to be described. The present article reports our efforts to determine such markers by employing DNA sequences coding for known and unknown proteins.

Hordein storage proteins are highly polymorphic polypeptides currently used to classify barley cultivars (Cooke and Morgan 1986; Kreis and Shewry 1992). These proteins are encoded by two families of genes that are responsible for the synthesis of complex mixtures of isophorms. Given the existence of polypeptide variability among barley varieties, we also believed it of interest to test the polymorphic state of the corresponding genes. The B- and C-hordeins are encoded by genetic loci designated *Hor2* and *Hor1*, respectively, which are located 8 cM apart on the short arm of chromosome 5 (Shewry et al. 1980). Hybridization studies have revealed homologies between B- and C-hordein genes (Forde et al. 1985).

Additional probes were corresponding to: (1) genes encoding for 5S-ribosomal RNA clustered on chromosome 2 (Kolchinsky et al. 1990); (2) DNA sequences encoding for leaf-specific thionins located on the short arm of chromosome 6 (Bohlmann and Apel 1987); (3) a barley embryo desiccation-induced gene; and (4) barley ubiquitin (Gausing and Barkardottir 1986). All of these probes are known to hybridize to a large number genes, even if not all of them reveal the same degree of polymorphism.

To increase the probability of finding the highest possible level of genetic polymorphism, four-cutter restriction enzymes were used to restrict the DNA of

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the barley varieties considered. These enzymes produce a greater number of gene fragments than six-cutter enzymes.

Materials and methods

Fifty-one barley varieties of different origin, growth habit and yield performances were chosen (Table 1). Young shoots from pot-grown plants were harvested, frozen in liquid nitrogen and stored at −70 °C if not used immediately. The DNA probes used to reveal RFLP polymorphisms were supplied by P. R. Shewry (pB11 and pcP387 corresponding to hordein B and C, respectively), K. Apel (DB4 corresponding to leaf-specific thionin), D. Bartels (p27–46, a barley embryo desiccation-induced cDNA clone), A. Kolchinsky (gene for 5S-RNA) and K. Gausing (ubiquitin cDNA clone pKG3730). The inserts were labelled using α-[^{32}P]-dCTP as the radioactive nucleotide (Amersham), following the random primer method of Feinberg and Vogelstein (1984). DNA samples from 10 g of young shoots were extracted and purified by CsCl. The genomic DNA (5 or 8 µg per gel sample) was digested overnight with the four-cutter restriction enzymes *Taq*I, *Rsa*I and *Hae*III. Four enzyme units per microgram DNA were utilized according to the supplier's instructions (Promega). The restriction fragments generated by the four-cutter enzyme digestions were separated on a denaturing 4% polyacrylamide gel and electro-blotted onto a nylon membrane (Hybond N, Amersham). Prehybridization of the membranes, hybridization in a rotating oven with the [^{32}P]-labelled probes and post-hybridization washes were performed after Gebhardt et al. (1989b). The membranes were autoradiographed for 7–14 days on Kodak X-ray film. Restriction fragments were identified and individually numbered for each combination probe/enzyme. The presence or absence of each fragment was scored as 1 or 0, respectively. A matrix based on these values was prepared (varieties × fragments) for each probe/enzyme combination. Data analysis was performed using the statistical programme GENSTAT 5 released on a computer back DEC-VAX-VMS 5.3. Similarity matrices were computed using the simple matching coefficients for each single probe/enzyme combination and for their most interesting double and triple combinations.

Results

Polymorphic probes

The high polymorphic RFLP probes described for plant DNA by Gebhardt et al. (1989a) and Görg et al. (1992) are anonymous clones found by chance. The final goal of our work was similar but, to restrict the number of probes to be tested in the barley fingerprint, we decided to use DNA sequences capable of revealing DNA fragments from multigenic families.

The probe pKG3730, which encodes ubiquitin, and the clone corresponding to 5S-ribosomal RNA showed less polymorphism than expected and consequently were discarded. Probe p27–46 revealed several restriction fragments, and the variability among varieties was of some interest in the fingerprint; curiously, this probe reveals a very atypical restriction pattern for the genotype Pilastro (data not shown). Probe DB4, which is homologous to genes encoding thionins, revealed complex patterns with a good polymorphism among varieties. The highest polymorphic probes were the

Table 1. Ear type, growth habit and origin of the barley varieties considered

Variety number	Name	Ear type[a]	Growth habit[b]	Country origin[c]
1	Jaidor	S	W	F
2	Panda	T	W	F
3	Trebbia	S	W	I
4	Aramir	T	S	NL
5	Express	S	W	F
6	Criter	S	W	F
7	Hulda	T	S	S
8	Roland	T	S	S
9	Etrusco	S	W	I
10	Timura	T	W	D
11	Selvaggio	S	W	I
12	Fiction	S	W	F
13	Kaskade	T	W	D
14	Dahlia	S	W	F
15	Georgie	T	S	GB
16	Marinka	T	W	GB
17	Flash	S	W	F
18	Porthos	T	S	F
19	Mirco	S	W	I
20	Aura	T	S	D
21	Arda	T	W	I
22	Gimpel	T	S	D
23	Tipper	T	W	GB
24	Cannon	S	W	I
25	Prisma	T	S	NL
26	Triumph	T	S	D
27	Gitane	T	S	B
28	Gavotte	T	S	F
29	Elan	S	W	F
30	Carina	T	S	D
31	Havila	T	S	NL
32	Pilastro	S	W	I
33	Atem	T	S	NL
34	Masto	S	W	D
35	Tapir	S	W	D
36	Torrent	T	W	GB
37	Thibaut	S	W	F
38	Crimont	S	W	B
39	Novoperga	S	W	D
40	Onice	S	W	I
41	Plaisant	S	W	F
42	Fleuret	S	W	F
43	Opale	S	W	I
44	Mette	T	S	S
45	Robur	S	W	F
46	Protidor	T	W	F
47	Tania	S	W	D
48	M. Otter	T	W	GB
49	Nico	T	W	I
50	Magie	T	W	F
51	Vogelsanger-Gold	S	W	D

[a] T, Two-rowed; S, Six-rowed
[b] W, Winter barley; S, Spring barley
[c] I, Italy; D, Germany; F, France; GB, Great Britain; S, Sweden; B, Belgium; NL, Holland

B- and C-hordein clones pB11 and pcP387. The restriction enzyme *Rsa*I gave the highest level of polymorphism with the probes tested, followed by *Taq*I and *Hae*III.

Fingerprinting

For each probe/enzyme combination, the presence or absence of individual polymorphic fragments was scored as described in the Materials and methods. The number of different patterns revealed by 12 single probe/enzyme combinations (probe pB11, pcP387, DB4, p27–46 and enzymes *Taq*I, *Rsa*I, *Hae*III) was determined. Ten of these probe/enzyme combinations are listed in Table 2. In addition, 31 of the 45 possible double combinations and 14 of the 120 possible triple combinations were considered. Double or triple combinations more adapted to varietal fingerprinting are also reported in Table 2. The minimum and maximum number of different fragments between any pair of varieties were computed for each single or multiple probe/enzyme combination.

Table 2 also reports the number of fragments revealed by each probe/enzyme combination. No single probe/enzyme combination succeeded in differentiating all of the varieties. However, probe pB11 coupled to enzyme *Rsa*I had a high discrimination power in revealing 44 different patterns out of the 51 varieties tested. An example of the RFLP patterns obtained with pB11/*Rsa*I is shown in Fig. 1.

The best double combinations were pB11/*Rsa*I + pcP387/*Hae*III and pB11/*Rsa*I + pcP387/*Taq*I: they distinguished, respectively, 50 and 49 different restriction patterns in the 51 varieties. The best triple combinations differentiated all of the varieties. The highest was the number of fragments by which one genotype was distinct from another, and the safest was its RFLP characterization. Effectiveness in discriminating barley varieties and reliability of RFLP analysis decrease correspondingly with a decrease in the number of polymorphic fragments. The maximum number of differentiating fragments generated by the single probes ranged from 4 (p27–46/*Taq*I) to 19 (pB11/*Rsa*I), while the mean number ranged from 2 to 10.

With double combinations the number of polymorphic fragments increased to 24–56, the maximum number of differing fragments ranging from 15 to 31 and the mean number from 8 to 15. A similar increase in effectiveness was noted in triple combinations. They showed 43 to 76 polymorphic fragments: the maximum

Table 2. RFLP pattern resolution in 51 barley varieties based on simple, double or triple probe/enzyme combinations

Probe/enzyme combination	Number of fragments	Number of differing patterns	Minimum-maximum number of different fragments between pairs of varieties
pB11/*Rsa*I	36	44	0–19
pcP387/*Taq*I	20	35	0–11
pcP387/*Hae*III	20	30	0–12
pcP387/*Rsa*I	19	28	0–16
DB4/*Rsa*I	11	28	0–10
pB11/*Taq*I	11	22	0–9
DB4/*Taq*I	9	20	0–7
pB11/*Hae*III	9	17	0–7
p27–46/*Taq*I	4	10	0–4
p27–46/*Hae*III	10	7	0–10
pB11/*Rsa*I + pcP387/*Hae*III	56	50	0–27
pB11/*Rsa*I + pcP387/*Taq*I	56	49	0–27
pB11/*Rsa*I	47	48	0–26
pB11/*Rsa*I + pB11/*Taq*I	47	48	0–25
pB11/*Rsa*I + p27–46/*Taq*I	40	48	0–22
pcP387/*Taq*I + p27–46/*Taq*I	24	48	0–15
pB11/*Rsa*I + pB11/*Hae*III	45	47	0–25
pB11/*Rsa*I + pcP387/*Rsa*I	55	47	0–31
pB11/*Rsa*I + DB4/*Taq*I	45	47	0–25
pB11/*Rsa*I + pcP387/*Taq*I + pcP387/*Hae*III	76	51	3–35
pB11/*Rsa*I + pcP387/*Hae*III + DB4/*Rsa*I	67	51	3–35
pB11/*Rsa*I + pB11/*Hae*III + pcP387/*Hae*III	65	51	3–34
pB11/*Rsa*I + pcP387/*Rsa*I + pcP387/*Hae*III	75	50	0–39
pB11/*Rsa*I + pB11/*Taq*I + pcP387/*Hae*III	67	50	0–33
pcP387/*Rsa*I + pcP387/*Taq*I + DB4/*Rsa*I	50	50	0–31
pcP387/*Rsa*I + pcP387/*Taq*I + p27–46/*Taq*I	43	50	0–26

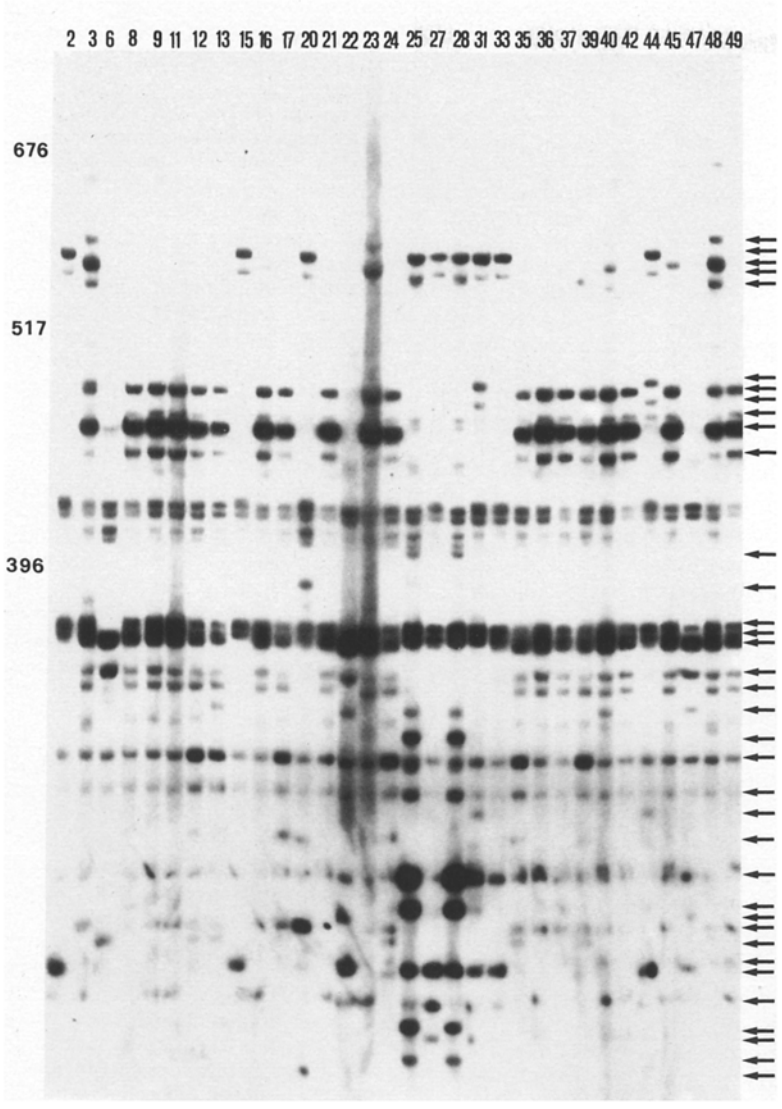

Fig. 1. RFLP patterns obtained with the four-cutter restriction enzyme *Rsa*I and the probe pB11. Note the occurrence of a middle-repeat hordein gene family. *Numbers* at the *top* of each lane refer to the reference numbers of the barley cultivars listed in Table 1. Molecular weight markers (in bp) are indicated on the *left*; fragments are indicated on the *right*

number of differing fragments ranging from 26 to 39 and the mean value varying between 13 and 20.

The single probe/enzyme combination pB11/*Rsa*I identified 44 RFLP patterns out of 51. Seven doublets of cultivars were not resolved: 'Masto'/'Torrent', 'Opale'/'Thibaut', 'Panda'/'Georgie', 'Roland'/'Etrusco', 'Nico'/'Fleuret', 'Mirco'/'Arda' and 'Magie'/'Vogelsanger-Gold'. All doublets could be differentiated by the RFLP patterns of an additional probe/enzyme. pcP387/*Taq*I discriminated between 'Mirco'/'Arda', 'Roland'/'Etrusco', 'Panda'/'Georgie' and 'Opale'/'Thibaut'; pcP387/*Hae*III between 'Nico'/'Fleuret' and 'Magie'/'Vogelsanger-Gold'.

Discussion

Barley varieties have been fingerprinted on the basis of morphological and physiological traits (Molina-Cano and Elena Rosselló 1978), hordein protein patterns (Cooke and Mogan 1986), hordeins and isoenzymes (Nielsen and Johansen 1986) and, recently, standard RFLP methods (Bunce et al. 1986). A comparison of all of these identification methods, including those of the present paper, indicates that the use of highly polymorphic RFLP probes is to date probably the most informative one. The system offers both simplicity and effectiveness: a single probe/enzyme combination (pB11/*Rsa*I) gave 44 different fingerprints out of 51 genotypes tested, while Cooke and Morgan (1986) classified 191 barley cultivars into 41 separate groups based on hordein patterns and Nielsen and Johnsen (1986) used 16 different isozymes to identify 63 patterns out of 66 varieties. As predicted, variability at the DNA level is higher than that found at the protein level; a probe corresponding to the single hordein locus *Hor2* used in combination with only one enzyme generated more polymorphisms than those generated

when the variability of all the polypeptides encoded by *Hor1*, *Hor2* and *Hor3* loci is taken into consideration.

Bunce et al. (1986) first proposed the use of B- and C-hordein probes to fingerprint barley cultivars with standard RFLP methods. Based on restriction patterns obtained by four-cutter enzymes, we found that the same probes are even more effective. It should, however, be possible to find in barley, as has been found in potato (Görg et al. 1992), additional probes that are effective in discriminating even a larger number of cultivars based on a single probe/enzyme combination.

Cultivars not identified by a single RFLP probe were distinguished in our study by a second probe/enzyme combination. The results indicate that most, if not all, barley varieties can be fingerprinted using a few probes and that by following such a procedure a classification key can be developed. Among the varieties tested some, such as 'Aura' and 'Atem', are easy to fingerprint, while other have similar RFLP patterns. The group of winter barley cultivars, 'Opale', 'Onice', 'Plaisant', 'Nico', 'Thibaut', 'Fleuret', 'Mirco', 'Arda', 'Trebbia', 'Flash' and 'Cannon' had a tendency to conserve a similar RFLP pattern with the probes tested. This may indicate the conservation which occurs in selected genotypes of chromosomal segments that are important for yield and adaptation, or the RFLP similarities may derive from the pedigree. In the case of 'Onice' and 'Opale' the pedigree comparison indicates that the second explanation is the more likely, indeed, the two cultivars derive from the same cross 'Perga' × 'Sam-Chio 36' (Sanguineti et al. 1979).

As suggested by Johns et al. (1983), probes corresponding to middle-repeat genes containing conserved domains flanked by high polymorphic sequences should be the most adapted to varietal fingerprints. In this sense, hordein and thionin gene-based probes confirm this prediction in barley.

Acknowledgements. The authors thank C. Peveri for assistance and Dr. A. Monetti for the computer analysis. This work was supported by Italian Ministry of Agriculture 'Progetto: Mappe genomiche'.

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