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R. Linacero · E. Freitas Alves
A.M. Vázquez

Hot spots of DNA instability revealed through the study of somaclonal variation in rye

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Abstract RAPD analysis was performed to assess DNA variation among rye plants regenerated from immature embryos and inflorescences. From the studied plants, 40% showed at least one variation, and the number of mutations per plant was quite high, ranging from 1 up to 12. On some occasions (2.9% of the scored bands) the modified band was observed in only one plant or in several but originated from the same callus (variable band). In other cases (5.25%) the same band varied in several plants obtained from different calli. We call these hypervariable bands and they could vary between plants belonging to different cultivars and/or with different origins, inflorescences or embryos. Thus, they must originate through independent mutational events. We assume that these bands represent hypervariable regions of the rye genome and so detect hot spots of DNA instability. Some of these bands proved to be unique sequences, others were present in a low copy number while the remaining ones were moderately or highly repetitive.

Key words Rye · DNA instability · Hypervariable sequences · Somaclonal variation · RAPD

Introduction

Over the past few years views concerning genome evolution have changed, and it is now accepted that the genome has an extremely high plasticity. Exchanges, deletions, insertions or amplifications are being recurrently produced, the new combination resulting in some cases in the appearance of new genes (Capy 1998). Moreover, it has been proposed that somatic genome plasticity, which is higher in plants than in animals, may improve individual adaptation (Buiatti and Bogani 1998). As in animals, genomic changes in plants could be related in some cases to different kinds of stress; these changes include amplifications (Cullis 1986) or transposable-element movements (McClintock 1984; Hirochika 1995). On the other hand, the presence in the genome of hypervariable sequences, in which the rate of mutation is higher than in other sequences, has also been described. Due to this fact, these sequences have been frequently used in several kinds of genome variability studies.

It is also known that plant tissue culture can promote mutational phenomena induced by stress imposed by in vitro conditions. The genetic variability originated is known as somaclonal variation (Larkin and Scowcroft 1981). Thus, even when in theory all the cells, organs or regenerated plants must be genetically identical to the original explant, and so identical to the plant from which this has been taken, different kinds of variations have been observed. Assuming that hypervariable regions must be affected by the mutation-inducing phenomena which seem to operate during plant tissue culture, it should be possible to identify the regions in the genome which most often vary by studying the somaclonal mutants. Hence, the increase of this genetic instability could be useful for detecting hypervariable sequences.

Rye (*Secale cereale L.*) is one of the species in which a high rate of somaclonal variation has been reported (Linacero and Vázquez 1993). In the present work, a molecular analysis of the regenerated plants of rye, using the RAPD technique (Williams et al. 1990), was performed to ascertain the type of sequences involved in this variation.

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R. Linacero · E.F. Alves · A. M. Vázquez (✉)
Departamento de Genética, Facultad de Biología,
Universidad Complutense, 28040 Madrid, Spain

Materials and methods

Plant material and DNA extraction

Three diploid cultivars of rye (Ailes, JNK and Merced) were used. Twenty three regenerated plants were obtained from immature inflorescences (cultivars Ailes and JNK) and 27 from immature embryos (cultivars Ailes and Merced) following Linacero and Vázquez (1990 and 1992 a, respectively), and DNA was isolated from their leaves when the plants reached maturity. In all the cases

in which DNA was analysed, the Dellaporta et al. (1983) isolation method was employed.

RAPD analysis

RAPD reactions were similar to those described by Williams et al. (1991) using primers from OPERON Technologies (Series OpB, OpC, OpF, OpG and OpS). After studying the pattern obtained from different primers, the following were used:

Embryo- and inflorescence-derived plants: OpF 02, 08, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20.
Embryo derived plants: OpB 04, 06; OpC 07, 15, 20; OpF 07; OpG 10; OpS 02, 03, 04, 07, 09, 10, 12, 18, 19.
Inflorescence-derived plants: OpF 03, 04, 05, 11; OpG 08, 09, 11, 13, 14, 15, 18, 19.

The 25-µl reaction mixtures contained 25–30 ng of DNA, 0.20 µM of primers, 100–200 µM of each dNTP and 1.25 U of Ampli*taq* DNA-polymerase Stofell fragment (Perkin Elmer) or Dinazyme (Finnzymes Inc.) with the corresponding buffer and $MgCl_2$. The PCR products were analysed by electrophoresis in a 2% TAE agarose gel and observed under UV light after staining with ethidium bromide. DNA from the variable bands was recovered from the gel (Geneclean Kit BIO 101 Inc.), and cloned into pCRII vector (Invitrogen).

Southern-blot hybridization

In some cases the PCR-amplified DNAs were run in a 1.7% agarose gel and then transferred to a nylon membrane (Hybond N^+, Amersham) as described by Sambrook et al. (1989). Cloned bands were used as probes and were labelled with digoxigenin (DIG-labelled-ddUTP Boerhinger Mannheim). Nylon filters were pre-hybridised and hybridised at 65 C in 5 × SSC, 0.1% laurylsarcosine, 0.02% SDS, 0.1% blocking reagent (Boehringer Mannheim). After hybridization the blots were washed twice for 5 min at room temperature in 2 × SSC, 0.1% SDS and then twice for 15 min in 0.1 × SSC, 0.1% SDS at 65 C. Detection of digoxigenin-labelled DNA was performed according to the protocol of the DIG-DNA labelling and detection Kit DNA colour reaction (Boehringer-Mannheim).

Genomic DNA, 10–15 µg, was digested with different restriction enzymes, electrophoresed in a 0.8% TAE agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham). DNA probes were labelled as mentioned above or with ^{32}PdCTP (ICN 3000 Ci/mol) using the random primer kit from Amersham. When DIG-labelled probes were used, Southern hybridizations were carried out at 65 C in 5 × SSC, 0.1% laurylsarcosine, 0.02% SDS, 1% blocking reagent. The washes and detection were performed according to the luminiscent manufacturer's indications (Boehringer Mannheim). The radiolabelled probes were hybridised in the presence of 5 × SSPE, 5 × Denhardt´s solution, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA at 62 C. Washes were performed at 62 C in 2 × SSPE, 0.1% SDS; 1 × SSPE, 0.1% SDS and 0.1 × SSPE, 0.1% SDS. Autoradiography was carried out at –70 C and the blots were exposed to Amersham MP hyperfilms with intensifying screens. Each blot was re-probed with the different probes. Between probes, the DNA blots were stripped by boiling in 0.5% SDS.

Results

Eighty different primers were used. From these we chose 40 whose pattern was reproducible and the number of amplified bands appropiate. In the case of the regenerated plants derived from inflorescences we assumed that, for a particular primer, the RAPD pattern obtained had to be the same in both the original plant from which we took the inflorescences and the regenerated plants obtained when we cultured them. Any change had to be due to mutation. In this case the pattern of the plant from which the inflorescences were taken was used as a control. In the case of plants regenerated from embryos, we could not compare the RAPD pattern of the regenerated plants with that obtained from the explant, because the embryo was used to obtain the callus from which the plants regenerated. However, all the plants obtained from the calli derived from one embryo had to have an identical pattern when RAPDs were performed using the same primer. Again, changes in the pattern had to originate from mutation. In these cases we considered that the most-frequent pattern between the plants derived from the same embryo had to be the normal one and the variable bands, the less frequent ones, originated by mutation.

Table 1 Variable bands.The primers used can be observed in the first column. The band name appears in column 2. The explant from which the plants regenerates are shown in column 3. In the 4th column the cultivar from which the explant was taken is shown

Primer	Band	Explant	Cultivar
OpF01	F01	Immature inflorescence	JNK
OpF08	F08	Immature embryo	Merced
OpG10	G10a G10b G10c	Immature embryo	Ailés Merced Merced
OpG13	G13	Immature inflorescence	Ailés
OpG16	G16	Immature embryo	Ailés
OpG17	G17	Immature inflorescence	Ailés
OpS06	S06	Immature embryo	Ailés

When a primer was used to amplify DNA from a particular set of plants (embryo-derived, immature inflorescence-derived or both) three different kinds of results were obtained:

No variation in the amplification pattern between the plants originating from the same callus.
One band varied in only one plant or in several plants originating from the same callus. We call these variable bands (Table 1).
The same band, with the same molecular weight, varied in several plants obtained from different calli. We call these hypervariable bands and they could vary between plants belonging to different cultivars and/or with different origins, inflorescences or embryos (Table 2).

Variable and hypervariable bands were named according to the primers used in each case. When more than one band was modified with the same primer we added a letter after the band name. Thus, F02a and F02b were two variable bands amplified with the OpF02 primer.

In all cases in which a variable or hypervariable band was detected, this band was cloned and was used as a

Table 2 Hypervariable bands. The primers used can be observed in column 1. The name of the hypervariable bands appears in column 2. Column 3 shows the type of explant from which the variable plants regenerate; note that one band could be variable in plants regenerated from both explants. Column 4 shows the cultivar to which the explants belong; note that one band could be variable in plants obtained from the same type of explant but from different cultivars. In column 5 the copy number of the band in the rye genome is shown

Primer	Band	Explant	Cultivar	Copy number
OpB04	B04a B04b	Embryo	Ailés, Merced	Single Single
OpC20	C20a C20b	Embryo	Merced	Single Moderately
OpF02	F02a	Inflorescence Embryo	Ailés Merced	Moderately
	F02b	Inflorescence Embryo	Ailés Ailés, Merced	Highly
OpF13	F13a	Inflorescence Embryo	Ailés, JNK Ailés, Merced	Low
	F13b	Embryo	Ailés, Merced	Moderately
OpF17	F17	Inflorescence Embryo	Ailés, Merced Ailés Merced	Moderately
OpF19	F19	Embryo	Merced	Low
OpF20	F20	Inflorescence Embryo	Ailés Ailés, Merced	Single*
OpG06	G06	Inflorescence Embryo	JNK Merced	Highly
OpS03	S03	Embryo	Merced	Low
OpS10	S10a S10b	Embryo Embryo	Ailés, Merced Ailés, Merced	Highly Moderately
OpS19	S19	Embryo	Ailés, Merced	Single

* Under high stringency conditions

probe to perform the corresponding Southern study, in order to confirm the results (Fig. 1). All the bands included in Table 1 hybridized with their corresponding probe, so we can affirm that when a band was missing or a new band appeared it was not due to any possible error. In the case of the hypervariable sequences (Table 2) the variable band was cloned from one plant and was used as a probe in all the cases in which this band was detected as variable. Thus, this probe was used to analyse all the plants amplified with the same primer which showed a variation in this particular band. In this way it was also possible to determine if a hypervariable band corresponded to the same sequence in all plants with the same modified pattern (Fig. 2). As the hybridization gave positive results, it was possible to conclude that the hypervariable bands contained the same sequences in all the cases referred to in Table 2.

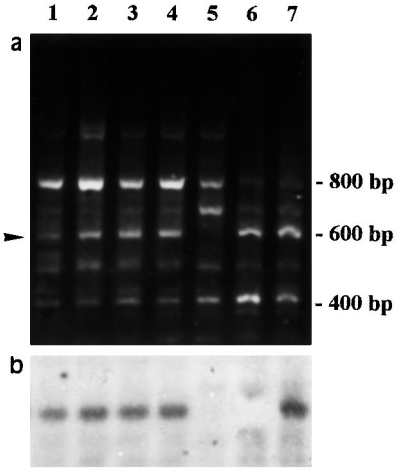

Fig. 1 **a** The RAPD pattern obtained from plants regenerated from one embryo callus with the OpS03 primer. The *arrow* indicates the polymorphic band. The band S03 was excised from the agarose gel and cloned. **b** Southern blot from the same gel, using the S03 band as a probe (DIG labelling). In plant 6, no hybridization was observed because the band was cut from the gel

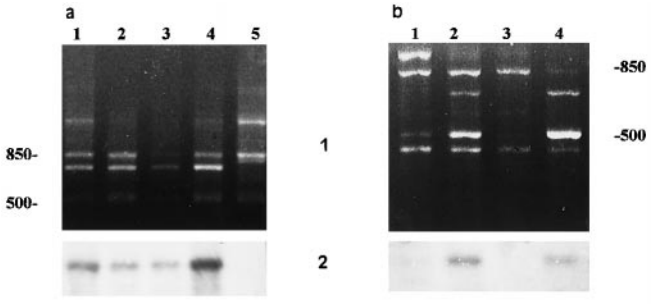

Fig. 2a, b Details of the RAPD patterns obtained with the OpF20 primer. **a,** *1* Plants regenerated from one immature inflorescence callus, cv Ailes. In plant 5, one band is missing. The variable band, F20, was isolated from the gel, cloned, and used as a probe in a Southern-blot hybridization performed with the transferred PCR amplified products (*2*). The absence of the band in the plant 5 is confirmed. **b,** *1* Plants regenerated from one immature embryo, cv Merced; one band was missing in plants 1 and 3. *2* Southern blot was performed on these PCR products using the F20 cloned band as a probe. The absence of the band is confirmed. The bands absent in both cases, **a** and **b**, correspond to the same sequence

Table 3 Plants which show some variation regenerated from immature inflorescences (**a**) or immature embryos (**b**). In column 1 the callus from which the plants regenerated is indicated. The plants shown in column 2, have been named according to the cultivar (A = Ailes, M = Merced and JNK). When more than one plant regenerated from the same callus presented the same mutation/s they are indicated in the same line. The following columns show if the plants presented the normal (–) or the abnormal (x) pattern for the bands indicated above. a,b or c correspond with the abnormal behaviour of the different bands amplified with the same primer (see text)

a

Callus	Plant	Band										N. changes
		F01	F02	F13	F17	F19	F20	G06	G13	G16	G17	
G	A 4	–	a, b	a, b	–	–	–	–	–	–	–	4
	A 2,3,5,6	–	–	–	–	–	x	–	–	–	–	1
	A 7	–	a	–	x	–	x	–	x	–	–	4
	A 8	–	–	–	–	–	x	–	–	x	–	2
	A 9	–	–	–	–	–	–	–	–	–	x	1
I	A 15	–	–	a, b	–	–	–	–	–	–	–	2
J	JNK 25	x	–	–	–	–	–	x	–	–	–	2
	JNK 23	–	–	–	x	–	–	–	–	–	–	1
K	JNK 22	–	–	a, b	–	–	–	–	–	–	–	2
	JNK 20	–	–	–	–	x	–	–	–	–	–	1

b

Callus	Plant	Band													N. changes
		B04	C20	F02	F08	F13	F17	F19	F20	G10	S03	S06	S10	S19	
A	M2	x	–	–	–	–	–	–	–	–	–	–	–	–	1
	M11	–	–	–	–	a, b	x	–	–	–	–	–	–	–	3
B	M15	x	–	b	–	–	–	–	–	–	–	–	–	–	2
	M27	–	–	a	–	–	–	x	–	c	x	–	a	–	5
C	M 29,30	–	a, b	–	x	a, b	–	–	x	–	x	–	b	x	9
	M 32	–	a, b	–	x	a, b	–	–	–	–	x	–	b	x	8
D	M 71	–	–	–	–	–	–	–	–	–	–	–	–	x	1
F	A 84	–	a, b	b	–	a, b	x	–	–	a, b	–	x	a, b	x	12
	A 97	x	–	–	–	–	–	–	–	a, b	–	x	–	–	4

A total of 305 bands were scored. Of these 2.95% (9/305) were variable and 5.25% (16/305) were hypervariable, i.e. 8.19% of the bands varied.

Some of the primers were used in all the plants studied (see Materials and methods). In these cases we could observe that when a particular primer detected variable plants among the ones originating from the inflorescence cultures it also detected variation among the embryo-regenerated ones. On the other hand, primers which did not detect variability in either type of plant coincided, with the exception of OpF08 which originated the band F08, with a variable band which appeared only in plants regenerated from one immature embryo.

Depending on individual cases, the variation observed in the pattern of amplification corresponded with the elimination of one band (F02a, F02b, F13a, F13b, G06 and S03), or the appearance of a new one (F17 and F20). On other occasions one band was eliminated and simultaneously a new one appeared (B04a and B04b; C20a and C20b). Finally, the variation in some plants implied the disappearance of one band, whereas in others the alteration corresponded with its presence (S10a, S10b and S19).

Forty per cent of the studied plants (21/58) showed at least one variation. The number of mutations per plant (the number of variable bands per plant) was also recorded. The observed data in the case of immature inflorescence-derived plants and immature embryo derived plants are shown in Table 3 a and b respectively. Most of the plants showed more than one change and, in some, the number of variations is very high.

Using the cloned bands corresponding to the hypervariable sequences as probes, we performed Southerns using genomic DNA isolated from plants growing in vivo, in order to ascertain the number of copies of these sequences present in the genome. Some proved to be unique sequences, others were present in a low copy number, while the remaining ones were moderately or highly repetitive (Table 2 and Fig. 3).

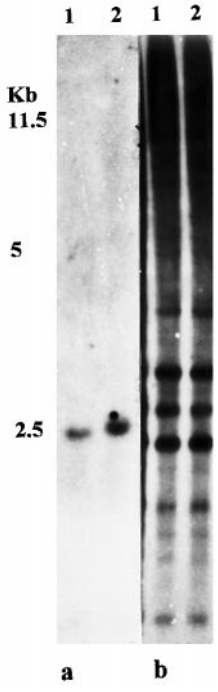

Fig. 3a, b Copy number of the hypervariable bands C20a (**a**) and F17 (**b**). DNA isolated from plants of the in vivo population of the Ailes (*1*) and Merced (*2*) cultivar was cut with *Eco*RV, run in a gel and transferred to a membrane to perform the Southerns. Cloned C20a and F17 bands were used as probes

Discussion

There are several molecular techniques used to detect DNA changes. Among them RAPDs have been employed and were applied in different cases to study DNA polymorphisms among and between populations, cultivars, etc. This technique has also proved to be quite a good method to detect the variation originated by tissue culture. Brown et al. (1993) found modifications in the RAPD pattern in rice plants derived from protoplasts, Godwin et al. (1997) in rice plants regenerated from mature seed, and Rani et al. (1995) in micropropagated plants of *Populus deltoides*. In other cases, however, no variation was found (Vallés et al. 1993; Fourré et al. 1997; Gallego et al. 1997). In the present work, as we have shown before (Linacero and Vázquez 1993), we found that somaclonal variation is a very frequent phenomenon in rye and now demonstrate that it can also be detected using molecular techniques. Thus, 42% of the studied plants presented variation and 8.19% of the scored bands were variable.

Concerning the sequences involved in the observed variation, we assumed that some of them represented hypervariable regions of the rye genome because several plants carried the same mutation, or at least had a mutation that modified a particular pattern in the same way. We confirm through hybridization that the variable sequence was the same in all these cases and we refer to these sequences as hypervariable bands. The presence or absence of these specific DNA fragments could be due to a mutation that modifies the DNA contained in the band, or else to the variation in nearby sequences which could be modified in such a way that the band disappears or a new band appears.

It is possible that a mutation occurred in an early step of callus formation, so that all, or at least part, of the callus cells carried the same mutation. If some regenerated plants were formed from these cells, all of them must carry the same modification. This could explain why from the same callus, we could regenerate plants which had the same variable band and for that reason we do not consider these changes as hypervariable because only one mutational event occurred. This is the case for band F08 which is variable in three plants all of which regenerated from callus C obtained from one embryo. In other cases the presence of several plants with the same modification, that is to say exactly the same polymorphic pattern has also been reported and this was interpreted in terms of the existence of a single somatic mutation in the cell from which the plants originated (Rani et al 1995). In the present study, however, we found the same variation in plants regenerated from different initial calli. Thus, independent mutational events must have occurred in exactly the same sequences; therefore the rate of mutation in them was quite high and we considered them hypervariable. It is interesting to note that even plants from different cultivars presented the same mutations.

The presence of loci with a high rate of mutation promoted by the in vitro conditions has been reported in some species (Xie et al. 1995). In rye, two independently regenerated plants presented a mutation for albinism affecting the same locus (Linacero and Vazquez 1992 b).

In some cases (bands S10a, S10b and S19) the variation was manifested as the loss of one band in some plants but the acquisition of the same band in others. This could mean that the same locus was being subjected to the mutational events and that the new alleles originated were already present in other plants of the cultivar. Consequently, in these cultivars some plants presented the band and the mutation caused its loss while in others the band was missing and the band appeared with the mutation. Using RFLPs, it has been described in soybean, that some regenerated plants belonging to a particular cultivar could present new generated alleles that were previously found in others; that is, the changes encountered in DNA from cultured cells are precisely those fragments found as RFLP alleles in other cultivars (Roth et al. 1989). In our case the situation may be similar. Thus, these particular loci could have a high rate of mutation but the alleles formed from these mutational events were the ones already present in other individuals in the in vivo populations. Hence, it might be possible that the mechanisms operating in nature which promote variability are the same ones that are inducing the variation found in vitro, and that the allele which appeared could be already present in the normal population. Thus, in vitro stress only increases the rate of mutation.

It was also noticeable that the number of mutations per plant was quite high. Indeed, even when we found some plants with only one mutation, the majority of the variable plants presented modifications in more than one band, the extreme being case represented by plant A84 which showed 12 variations. Plants with more than one

mutation have been previously reported; the average frequency of variation per plant in maize is 1.2 as referred to by Edallo et al. (1981) or 1.3 according to Lee and Phillips (1987). In rye this same phenomenon has also been reported (Linacero and Vazquez 1993).

Hypervariable sequences are in many cases repetitive or highly repeated, as for instance microsatellites or the r-DNA intergenic regions. The search for sequences with a high rate of mutation that involve other types of sequence is necessary if we are interested in quantifying the variation which occurs in sequences involved in phenotypic expression. RAPDs could, at least in theory, amplify any part of the genome, due to the fact that the oligonucleotides used as primers in the amplification reaction might have their sequence represented all around the genome. When we studied the copy number of the hypervariable bands we found that all kinds of sequences were represented, from single to highly repetitive; thus, our hypervariable sequences are not restricted to the repetitive ones. It could be argued that as the regenerated plants we used were phenotypically normal they did not carry the mutation on regulatory or coding sequences, because otherwise the phenotype would have been affected. However, we must be aware of the fact that in rye, as previously stated (Linacero and Vazquez 1993), most of the mutations with a manifestation in the phenotype are recessive, so that although the regenerated plants looked normal the abnormalities appeared in their descendants. Therefore, it could be possible that the modification in these hypervariable sequences promoted visible changes when present in homozygosis.

It is also noticeable that once one primer amplified a polymorphic band in more than one plant, the possibility of finding more modified plants using this primer was significantly increased. Thus, when a primer detected a hypervariable band in the plants regenerated from immature inflorescences, the same primer detected this band modified in the embryo-derived plants. This again indicates that hypervariable sequences are being detected and that at least some mutations are not produced at random in the genome, but are more frequent in the sequences amplified with some particular primers. We assume that by using these primers in rye we will be successful in finding variation among any regenerated plants.

The analysis of the hypervariable bands should indicate which the sequences are involved in the variation observed and probably give some indication of the mutation mechanisms implicated in the appearance of the modified bands. Moreover, at least in rye, tissue culture could be an important tool to study the regions in the genome which are more susceptible to modification; that it to say, the hypervariable DNA sequences. In these terms, the hypervariable bands or their flanking sequences represent hot spots of DNA instability.

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