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VARIATION OF RETROTRANSPOSON MOVEMENT IN CALLUS CULTURE AND REGENERATED SHOOTS OF BARLEY

Sibel Yilmaz, Nermin Gozukirmizi
Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, Istanbul, Turkey
Correspondence to: Sibel Yilmaz
E-mail: sibelyilmaz22@hotmail.com

ABSTRACT

In this study, tissue-culture-induced BARE1 (BArley Retrotransposable Element-1) retrotransposon movements were investigated in barley calli and regenerated shoots, using the Inter-Retrotransposon Amplified Polymorphism (IRAP) technique. IRAP analysis was carried out on 30-, 60-, 90-day-old calli and shoots regenerated from them. Samples from calli and regenerated shoots originating from same embryo were accepted as a group. Two sample groups originating from two different embryos were analyzed. DNAs from three mature embryos were used as control samples. The IRAP analysis of the control samples showed identical IRAP profiles of 12 bands for all three mature embryos, suggesting a lack of natural polymorphism between the tested individuals. The IRAP profiles of the two analyzed groups were different from those of the control embryo. While the samples from the first group had 14 IRAP bands, those of the second group had 16. An important feature observed for the samples from both groups was that they possess homomorphic IRAP profiles within the group. The samples from the first group had four new and two missing bands when compared with the IRAP profile of the control embryos. Similarly, the samples from the second group had four novel bands when compared with the embryos' band profile. The similarity rates, calculated by Jaccard's coefficient, were found to be 62.5 %, 75 % and 86.6 % between embryo–first-group, embryo–second-group and first-group–second-group, respectively. The obtained results indicated that the applied tissue culture conditions could efficiently induce BARE1 movements. The observed homomorphic IRAP profiles within the group of samples consisting of calli and regenerated shoots originating from the same embryo further suggests that BARE1 activation and movements possibly occur at early stages of callus initiation after in vitro cultivation of embryos. In addition, the difference of the IRAP detected polymorphism of BARE1 between the two analyzed groups indicates that similar tissue culture conditions could induce diverse rates of these BARE1 movements.

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Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; AFLP: Amplified Fragment Length Polymorphism; IRAP: Inter Retrotransposon Amplified Polymorphism; ISSR: Inter Simple Sequence Repeat; LTR: Long Terminal Repeats; MS: Murashige and Skoog; RAPD: Randomly Amplified Polymorphic DNAs; SSAP: Sequence Specific Amplified Polymorphism.

Introduction

Retrotransposons are ubiquitous and dynamic elements in the genome. In the plant kingdom, especially in cereals, they comprise up to 80 % of the genome (4, 41). They use an RNA intermediate mechanism for transposition. Because of their copy–paste transposition, they cause genome expansion (12, 31, 38). Considering their transposition mechanism and structure, they are thought to resemble retroviruses (18, 27, 28). Their new copies can insert themselves into near or within genes in a head-to-head, tail-to-tail or head-to-tail orientation. Therefore, they can cause altered gene products, frame-shift mutations, reduction of transcription level or even silencing of genes (9). Due to their dynamic feature, they are accepted as an important reason for genome evolution and speciation (5). Since retrotransposon insertions are irreversible, they are considered useful genetic elements in phylogenetic studies (20). Due to their variation capacity between species, retrotransposons are usually studied for detection of genetic relationships between varieties and related species (1, 2, 3, 25, 29, 32, 39).

Retrotransposons are classified into two subclasses, with respect to the existence of an LTR (Long Terminal Repeat) sequence (LTR-retrotransposons) or not (non-LTR retrotransposons). In plants, the most abundant group of retrotransposons are LTR-retrotransposons. They have a conserved domain that encodes proteins required for transposition, between LTR sequences (21, 33). *Gag* and *pol* genes are the components of this domain. While *gag* encodes capsid proteins, *pol* encodes protease, reverse transcriptase, integrase and RNaseH. The orientation of *pol* classifies LTR-retrotransposons as copia-like and gypsy-like (5, 19, 30, 34).

Although retrotransposons have transpositional potential, generally a high percentage of retrotransposons are inactive during plant development (15). However, they may be activated by different biotic or abiotic stress conditions like wounding, pathogen attack, tissue culture conditions, different drug or chemical applications (6, 11, 14, 16, 18, 40).

BARE1 is a copia-like retrotransposon, which was first characterized in barley (23). It constitutes nearly 7 % of the barley genome and is dispersed on all chromosomes (33). It was

found to be widely distributed within *Triticeae*. Leigh et al. (21) demonstrated that *BARE1* is the most active retrotransposon in barley and is an important factor for its genome organization.

Inter-Retrotransposon Amplified Polymorphism (IRAP) is an efficient technique to determine genome polymorphism generated by retrotransposon movements (17). To date, IRAP has generally been used to investigate retrotransposon derived polymorphism between species (13, 24, 37). However, it could be efficiently applied for analysis of the retrotransposon movement rates within a species and varieties to study the effects of tissue culture and evaluate the rate of induced somaclonal variation (7, 8).

In this study, we applied the IRAP technique to study the polymorphism and *BARE1* retrotransposon movement in barley calli and regenerated shoots obtained after callus induction from mature embryos and different periods of *in vitro* cultivation.

Materials and Methods

Callus culture and regeneration

Barley (*Hordeum vulgare* cv. Zafer-160) seeds were surface-sterilized with commercial bleach for 20 min and rinsed with sterile dH_2O three times for 10 min. After sterilization, mature embryos were removed from seeds and dipped in absolute ethanol for 30 s. Ethanol was removed and seeds were rinsed with sterile dH_2O three times for 1 min. Embryos were dried on sterile filter paper and cultured on MS medium (3 % sucrose, 0.9 % agar, pH 5.7) supplemented with 3 mg/L of 2,4-D (Sigma, D7299). Each embryo was given a number and incubated at 25 °C ± 2 °C in complete darkness for 30 days. At the end of the incubation time, each callus was cut into three pieces and the first piece was used for genomic DNA isolation, the second one was sub-cultured at the same conditions for another 30 days, and the third one was induced for shoot regeneration in MS0 medium at long-day conditions (16 h light/8 h dark, 25 °C ± 2 °C). We constructed two groups of test samples. Each group consisted of 30-, 60-, and 90-day-old calli and regenerated shoots which originated from the same embryo (**Fig. 1**).

Genomic DNA isolation

Genomic DNAs from mature embryos, calli and *in vitro* shoots were isolated according to Rogers and Bendich (26). The quality of DNAs was controlled with a 1 % agarose gel and the quantity of DNAs was measured spectrophotometrically.

Inter-Retrotransposon Amplified Polymorphism (IRAP) PCR

IRAP was performed with *BARE1* specific primers (LTR6149→ 5' CTCGCTCGCCCACTACATCAACCGCGTTTATT 3' and 5'LTR2← 5' ATCATTCCCTCTAGGGCATAATTC 3'). Primer sequences were obtained from Teo et al. (35). Amplification reactions were carried out in a 20 µL reaction volume containing 9.9 µL of nuclease-free dH_2O, 2.0 µL of 10X buffer (1X), 2.0 µL of 25 mmol/L $MgCl_2$ (2.5 mmol/L), 2 µL of 10 mmol/L (2.5 mmol/L each) dNTP mixture (1 mmol/L), 1.6 µL of primer (8 pmol, 0.8 µmol/L), 2 µL of 10 ng/µL template genomic DNA (20 ng, 1 ng/µL) and 0.5 µL of 5 U/µL *Taq* (*Tsg* polymerase, BioBasic) DNA polymerase (2.5 U, 0.125 U/µL). The amplification conditions consisted of one initial denaturation step at 94 °C for 2 min followed by 30 cycles of 94 ºC for 30 s, 59 ºC for 30 s and 72 °C for 3 min. The reactions were completed by a final extension step at 72 °C for 7 min.

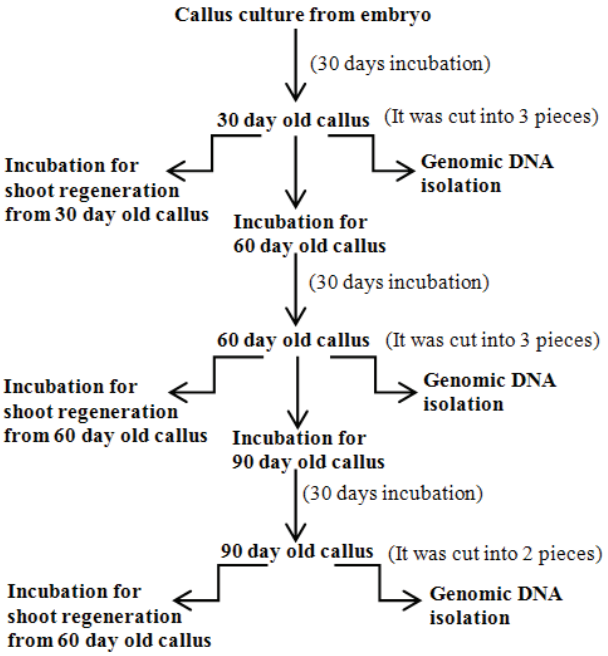

Fig. 1. Construction of the test group (scheme).

Evaluation of PCR products

Polyacrylamide gel electrophoresis was employed to separate PCR products. Ten-microliter aliquots of IRAP-PCR products were mixed with 2 µL of 6X loading buffer (10 mmol/L Tris-HCl, 60 mmol/L EDTA, pH 8.0, 0.3 % bromophenol blue, 60 % glycerol) and resolved in 8 % non-denaturing polyacrylamide (29:1 Acrylamide:Bis) gels at 200 V for 6 h in 1X TBE buffer (90 mmol/L Tris-borate and 2 mmol/L EDTA, pH 8.0). A molecular weight marker (GeneRuler™ 1 kb DNA Ladder, SM0312, Fermentas) was also loaded to determine the size of amplicons. Gels were stained in 1X TBE buffer containing 0.5 µg/mL ethidium bromide for 15 min. After staining, gels were rinsed with distilled water and photographed on a UV transilluminator. Well-resolved bands were scored as a binary value, [1] for presence and [0] for absence. The binary matrix [1/0] was used to calculate the similarity between embryo and samples. Jacquard's similarity index was calculated using the formula: $N_{AB}/(N_{AB} + N_B + N_A)$; where N_{AB} is the number of bands shared by two samples, N_A represents amplified fragments in sample A, and N_B represents amplified fragments in sample B.

Results and Discussion

To find the effects of tissue culture conditions and culturing time on *BARE1* movement in barley calli and regenerated shoots, we used the IRAP technique. First, three randomly selected

TABLE 1

IRAP fingerprint analysis results

Group	Total band number	Homomorphic band number shared with:		Polymorphic band numbers		
		Embryo	The other group	New	Missing	Specific
First group	14	10	13	4	2	1
Second group	16	12	13	4	0	1

mature embryos were analyzed and their IRAP profiles were compared with each other to identify whether there are natural IRAP polymorphisms of *BARE1* between the individual seeds used in this study. A total of 12 bands, under 1000 bp, were observed at IRAP-PCR of each mature embryo (**Fig. 2**, Lane 1–3). All of these bands were homomorphic. This result indicated that there are no natural polymorphisms between individual embryos with respect to *BARE1* transpositions.

The IRAP analysis of the first group of samples from 30-, 60-, 90-day-old calli and regenerated shoots showed 14 homomorphic bands present in the IRAP profiles of each sample in the group (**Fig. 2**, Lane 4–9). The comparison of the IRAP profile of the first group of samples with the IRAP profile of the control mature embryo samples revealed the appearance of four new bands (**Fig. 2**, arrows **a** and **c**) and two missing bands (**Fig. 2**, arrows **b**). While three of these four new bands (arrows **a**) were slightly observed in the second group (**Fig. 2**, Lane 10–15), the other one (arrow **c**) was only specific for the first group (**Table 1**).

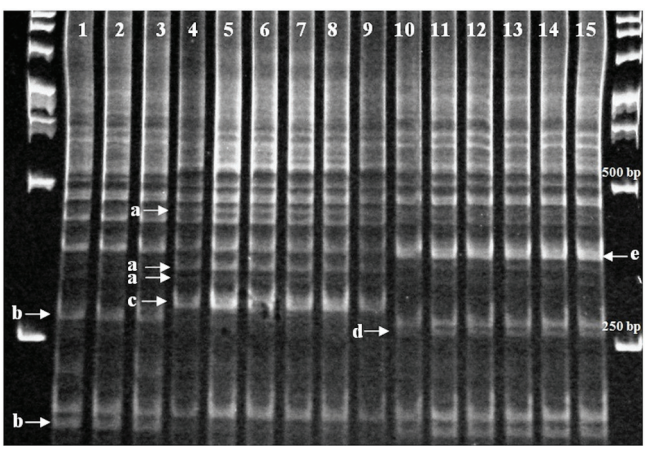

Fig. 2. IRAP profiles for *BARE-1*. Lane 1–3: mature embryo (control); Lane 4–9: first group, Lanes 4, 5, 6: calli originating from the same embryo (30-, 60-, 90-day-old, respectively), and Lanes 7, 8, 9: regenerated shoots originating from these calli, respectively; Lane 10–15: second group, Lanes 10, 11, 12: calli originating from another embryo (30-, 60-, 90-day-old, respectively), and Lanes 13, 14, 15: regenerated shoots originating from the second group's calli. Arrows indicate the polymorphic bands.

The IRAP profile of the second group (**Fig. 2**, Lane 10–15) was different from those of the first group. A total of 16 IRAP bands were observed in the profile of the second group (**Table 1**). The bands were homomorphic and present in the profiles of all samples from the group. In contrast to the first group, the profiles of the second group of samples did not have any missing band when compared to the IRAP profiles of embryos. The IRAP profiles of the second group had four new bands, three of which also existed in the first group (**Fig. 2**, arrows **a**), while the other one was specific for this group (**Fig. 2**, arrow **d**). In addition to these results, we also observed that the amplification degree of the band in the second group was higher than those of the first group and the control embryo samples (**Fig. 2**, arrow **e**). This indicates that there might be some retrotransposon movement in the second group which causes the same length fragments which already exist in the band profile. The calculation of Jacquard's similarity index (**Table 2**) and data analysis showed different rates of polymorphism between the studied group of samples: 37.5 % between the embryo and the first group, 25 % between the embryo and the second group, and 13.4 % between the first and the second group.

TABLE 2

Similarity rates (%) between embryo (control) and groups

	embryo	1st group	2nd group
embryo	-	62.5	75
1st group		-	86.6
2nd group			-

In our previous study, we tested *BARE1* directed somaclonal variations in 30-, 45- and 60-day-old calli, using the IRAP technique with 25 primer combinations, and the polymorphism rates were between 14 % and 25 % (8). However, in that study the analyzed samples were a bulk of calli from each age. In the present study, the samples from each group were analyzed separately, which allows to more precisely point out the period of *in vitro* cultivation when *BARE1* movements occur. The lack of IRAP polymorphism between calli and shoots obtained after different periods of *in vitro* cultivation suggests that most likely *BARE1* activation and transposition happen at a very early stage of *in vitro* cultivation of mature embryos and callus initiation. The observed different polymorphism rate between the two test groups indicated that the applied tissue culture have a different effect on the individually cultivated mature embryos.

Insertional activities of retrotransposons are known as one of the reasons of somaclonal variations during the tissue culture process (8, 36, 40). Most of the genetic and epigenetic changes in the genome occur during the dedifferentiation process. This process begins at the earlier stages of the callus culture. Polyploidization, one of the reasons of somaclonal variations, was previously reported to happen after the 3rd day of callus culture (10). In our study, polymorphic bands were observed at the 30th day of callus culture and persisted through

the culturing time. This indicated that the insertional activity of *BARE1* initiated before the 30th day, at the earlier steps of tissue culture, and this result is parallel with previous reports mentioned above.

Most of the plant retrotransposons lost their activity throughout the evolutionary process by various mechanisms such as methylation of the promoter region and loss of an internal domain. However, they are able to regain their activity by some stress factors like tissue culture conditions. Some of the tissue culture stress factors are synthetic medium components, hormones and wounding of explants. One of the most preferred hormones to induce callus formation is 2,4-D, and it is known to cause a dramatic change in cytosine methylation (22). Previously, some plant retrotransposons have been shown to use cytosine methylation for inactivation and to be activated again during protoplast culture (4, 14). In this study, we also used the 2,4-D hormone for callus formation. This might suggest that methylation alterations in the callus cause *BARE1* activation in callus culture.

Conclusions

Our results prove that tissue culture conditions cause *BARE1* retrotansposon movements. Because all the polymorphic bands were observed at the 30th day, we can conclude that *BARE1* retrotransposon movements possibly occur at the earlier steps of callus formation in the time of dedifferentiation. While the band patterns of calli of a different age which belong the same group were identical, equally old calli from different groups had different band profiles. This might indicate that the effect of tissue culture on *BARE1* movement is not the same in every individual. Hence, each calli originating from a different embryo has its own genome fate during the tissue culture process. Therefore, variations of the genome of calli cells via retrotransposon movements may contribute to diversify plant phenotypes.

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