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Variations in *BARE-1* insertion patterns in barley callus cultures

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ABSTRACT. The stability of aging barley calli was investigated with the barley retroelement 1 (*BARE-1*) retrotransposon specific inter-retrotransposon amplified polymorphism (IRAP) technique. Mature embryos of barley (*Hordeum vulgare* cv. Zafer-160) were cultured on callus induction MS medium supplemented with 3 mg/L 2,4-D and maintained on the same medium for 60 days. Ten IRAP primers were used in 25 different combinations. The similarity index between 30-day-old and 45-day-old calli was 84%; however, the similarity index between mature embryos and 45-day-old calli was 75%. These culture conditions caused *BARE-1* retrotransposon alterations to appear as different band profiles. This is the first report of the use of the IRAP technique in barley in an investigation of callus development.

Key words: *BARE-1*; *Hordeum vulgare* L.; Tissue culture; Inter-retrotransposon amplified polymorphism

INTRODUCTION

Somaclonal variation is used to describe the occurrence of genetic variants derived from *in vitro* procedures; it is also called culture-induced variation (Larkin and Scowcroft, 1981). Many plant biotechnology applications use plant tissue culture as a tool, and the unpredictable nature of these variations represents a serious problem, especially for the commercial applications of such technology. Such variations in micropropagated material, such as genetic and epigenetic modifications, need to be characterized (Santos et al., 2008).

Hirochika (1993) showed that enhanced transcription of retrotransposons occurs in *Nicotiana* cells. LTR-retrotransposons, flanked by long terminal repeats (LTR), are one of the subtypes of retroelements and transpose in a replicative manner using an RNA intermediate. This means that each transposition event creates a new copy of the transposon while the original copy remains intact at the donor site (Grzebelus, 2006). They can reach high numbers and may be one of the major contributors to large genome size and variation (Vitte and Panaud, 2005). Detailed information about retrotransposon structure and applications of retrotransposons were reviewed elsewhere (Zou et al., 2009). The first complete retrotransposon to be sequenced is barley retroelement 1 (*BARE-1*). The *BARE-1* family is present in 14,000 full-length copies dispersed on all chromosomes, which constitute nearly 2.9% of the total size of the barley genome. The first full-length *BARE-1* element, named *BARE-1a*, is 12,088 bp long, but it contains a 3135-bp insertion in its 3'LTR (Manninen and Schulman, 1993). Several molecular marker systems based on retrotransposons have been developed. All rely on the principle that a joint is formed, during retrotransposon integration, between genomic DNA and the retrotransposon. These joints may be detected by amplification between a primer corresponding to the retrotransposon and a primer matching a nearby motif in the genome (Schulman et al., 2004). Distribution and movement of *BARE-1* elements have been used to assess polymorphism between barley genotypes (Waugh et al., 1997) and between tissue culture-regenerated barley plants (Li et al., 2007).

The inter-retrotransposon amplified polymorphism (IRAP) method (Kalendar et al., 1999) detects retrotransposon insertional polymorphisms by amplifying the portion of DNA between two retroelements. It uses one or two primers pointing outwards from an LTR and amplifies the tract of DNA between two nearby retroelements. IRAP can be carried out with a single primer matching either the 5' or 3' end of the LTR but oriented away from the LTR itself, or with two primers. The amplification products are generally resolved on wide-resolution agarose gels. The amplified fragments range from under 100 bp to over several kilobase pairs and are generally resolved by electrophoresis (Schulman et al., 2004). Due to the high copy number of *BARE-1* LTRs in the genome, IRAP generates too many bands to be easily scored by agarose gel, particularly with a 3'LTR primer; fragments can be resolved by polyacrylamide gel electrophoresis (PAGE) (Leigh et al., 2003). Polymorphism is easily detected by the presence or absence of the polymerase chain reaction (PCR) product, and the lack of amplification indicates the absence of the retrotransposon at the particular locus. This system was used to distinguish barley varieties (Kalendar et al., 1999), to map defense-related genes in barley (Manninen et al., 2000), to study grass genome evolution (Vicient et al., 2001), to detect retrotransposon

integration events in allopolyploid *Spartina anglica* (Baumel et al., 2002), to characterize somaclonal variation in banana (Muhammad and Othman, 2005), and recently in barley (Campbell et al., 2011), to investigate genetic relationships of *Diospyros kaki* and related species (Guo et al., 2006), as well as to group *Fusarium oxysporum* f. sp. *lactucae* isolates (Pasquali et al., 2007), to assess stability of pea long-term cultures (Smykal et al., 2007), and to evaluate evolutionary relationships among accessions of *Aegilops tauschii* (Saeidi et al., 2008). In the present study, we aimed to detect any possible *BARE-1* insertion variations among calli of different stages (30 days old, 45 days old and 60 days old) and mature embryo tissue. For this purpose, we employed the IRAP technique with 10 LTR primers in 25 different combinations, and the results were evaluated with the PAGE resolution technique.

MATERIAL AND METHODS

Mature embryos, which were aseptically removed from surface-sterilized barley (*Hordeum vulgare* cv. Zafer-160) seeds, were cultured on MS medium (3% sucrose, pH 5.7, 0.9% agar) supplemented with 3 mg/L (4.53 μM) 2,4-D (Sigma, D7299). Cultures were incubated at 25 ± 2°C in complete darkness and were sub-cultured at 3-week intervals.

Genomic DNA samples from 50 mature embryos (approximately 60 mg) and 30-, 45- and 60-day-old calli (pools of calli each comprising 3 randomly selected pieces of calli) were isolated using an isolation kit (EZ-10 Spin Column Genomic DNA Kit, Plant Samples, BS426, BioBasic). Quantity and quality of genomic DNA were measured spectrophotometrically.

A total of 25 combinations with 10 LTR primers were tested. 3'LTR, LTR6149, 5'LTR1, 5'LTR2, and LTR6150 primer sequences were obtained from Teo et al. (2005). Direct *BARE-1* and inverse *BARE-1* primer sequences were obtained from Schulman et al. (2004). However, three additional primers (PRM3F, PRM3(2)F and PRM3R) were designed from *BARE-1a* sequence (GenBank accession #Z17327) using the Primer3 software (http://frodo.wi.mit.edu/primer3). Primer sequences and melting temperatures are shown in Table 1. Amplifications were performed in a total volume of 20 µL containing 9.9 µL sterile distilled water, 2.0 µL 10X buffer (1X), 2.0 µL 25 mM $MgCl_2$ (2 mM), 2 µL 10 mM (2.5 mM each) dNTP mixture (1 mM), 0.8 µL of each primer (8 pmol, 0.4 µM), 2 µL 10 ng/µL template genomic DNA (20 ng, 1 ng/µL) and 0.5 µL 5 U/µL *Taq* (*Tsg* polymerase, BioBasic) DNA Polymerase (2.5 U, 0.125 U/µL). The values given in parentheses were the final concentrations. IRAP-PCR was carried out using a Creacon-TC-Y thermal cycler. The amplification conditions were one initial 2-min step at 94°C followed by 30 cycles at 94°C (30 s), variable (30 s) and 72°C (3 min); the reactions were completed by a final extension step of 10 min at 72°C. A 10-µL aliquot of IRAP-PCR products was mixed with 2 µL 6X loading buffer (10 mM Tris-HCl, 60 mM EDTA, pH 8.0, 0.3% bromophenol blue, 60% glycerol) and resolved on a 8% nondenaturing polyacrylamide (29:1, acrylamide:bis) gel at 200 V for 6 h in 1X TBE buffer (90 mM Tris-borate and 2 mM EDTA, pH 8.0). Gels were stained in 1X TBE buffer containing 0.5 µg/mL ethidium bromide for 15 min. A molecular weight marker (GeneRuler™ DNA Ladder Mix, SM0331, Fermentas) was also loaded to determine the size of amplicons. After staining, gels were rinsed with distilled water, photographed on a UV transilluminator and scored visually.

Table 1. List of primers used in this study and related information.

Name and orientation	Sequence 5' - 3'	T_m (°C)
3'LTR →	TGTTTCCCATGCGACGTTCCCCAACA	65.1
LTR6149 →	CTCGCTCGCCCACTACATCAACCGCGTTTATT	68.4
Direct *BARE-1* →	CTACATCAACCGCGTTTATT	53.7
PRM3F →	AGATGCGCTTCTATCCCTGA	57.8
PRM3(2)F →	TCCGTCTCTCTTGCTGGATT	57.8
5'LTR1 ←	TTGCCTCTAGGGCATATTTCCAACA	60.3
5'LTR2 ←	ATCATTCCCTCTAGGGCATAATTC	58.5
LTR6150 ←	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA	67.9
Inverse *BARE-1* ←	GCCTCTAGGGCATAATTCCAAC	60.0
PRM3R ←	AGAGGAAAAACCGTGGAGGT	57.8

T_m = melting temperature.

Primer combinations that failed to generate clear fingerprints were ignored. 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 by Jaccard's coefficient (Jaccard, 1908) among samples (embryo, and 30-, 45-, and 60-day-old calli). Due to the dominant nature of IRAP (Baumel et al., 2002), Jaccard's coefficient, which is one of the appropriate indices for dominant markers, was chosen. 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 2 samples, N_A represents amplified fragments in sample A, and N_B represents amplified fragments in sample B.

RESULTS

Barley (*H. vulgare* cv. Zafer-160) mature embryo and calli at different stages (30-, 45- and 60-day-old calli) were analyzed by the IRAP technique using LTR primers designed from the *BARE-1* sequence. A total of 25 combinations (Table 2) with 10 primers were tested. Due to variable melting temperatures (T_m, 53.7-68.4°C) of primers, we tested several annealing temperatures (T_a) such as 60-51°C. However, too many, probably non-specific amplicons, were produced at the T_a of 51°C. Therefore, for IRAP reactions using primers with lower T_m, T_a was adjusted to 52°C; using primers with higher T_m, T_a was adjusted to 59°C. These T_a values were different of some of previous results (Kalendar et al., 1999; Leigh et al., 2003; Vukich et al., 2009). However, some authors reported good results at the same or lower T_a (Muhammad and Othman, 2005; Teo et al., 2005). We adjusted the enzyme concentration to 2.5 U (0.125 U/µL). This concentration was higher than the recommended value (Kalendar and Schulman, 2006), but we could not amplify strong bands with lower amounts (1.5, 2.0 U) of enzyme. The quality of the enzyme may be the reason. We also tried to separate amplicons on medium-scale (14 cm in length) horizontal electrophoresis system, but we failed to analyze bands. Length of the electrophoresis apparatus and the quality of agarose may have caused poor resolution.

Eleven of 25 primer combinations failed to amplify any scorable bands. These primer combinations either gave a smear or very faint bands. Some of them amplified too many and also too large bands (over 10 kb), which could not be separated on polyacrylamide gels, particularly 3'LTR primer combinations. For instance, 3'LTR (T_m = 65.1°C) + PRM3R (T_m = 57.8°C) combination produced a total smear on the gel. However, the longest primers LTR6149 (32 mer) and LTR6150 (35 mer) produced good results. Despite their different melting temperatures, 3'LTR (T_m = 65.1°C) + 5'LTR2 (T_m = 58.5°C) and PRM3(2)F (T_m = 57.8°C) + LTR6150 (T_m = 67.9°C) combinations yielded scorable bands.

Table 2. All primer combinations tested in the study in terms of quality of amplification bands.

	5'LTR1	5'LTR2	LTR6150	Inverse *BARE-1*	PRM3R
3'LTR	N	S	N	N	N
LTR6149	S	S	S	S	S
Direct *BARE-1*	N	S	S	S	N
PRM3F	S	S	S	N	S
PRM3(2)F	N	N	S	N	N

N = non-scorable; S = scorable.

Fourteen of 25 combinations gave a total of 197 bands that were discrete, sharp and strong enough to be scored (Table 3). Sixty-six of 197 bands were polymorphic between embryo and calli. Only the PRM3F + 5'LTR2 primer combination produced a total of 10 bands but failed to detect any retroelement polymorphism. The degree of polymorphism of the other 13 combinations ranged from 9 to 53%. Results of 3'LTR + 5'LTR2 (110-620 bp) and PRM3F + PRM3R (170-1000 bp) primer combinations are presented in Figure 1A and B.

Table 3. Inter-retrotransposon amplified polymorphism (IRAP) band profiles of primer combinations.

Primers	T_a	Total	Monomorphic	Polymorphic (%)	Range of band size (bp)
3'LTR + 5'LTR2	59	26	14	12 (46%)	110-620
LTR6149 + 5'LTR1	59	15	7	8 (53%)	180-480
LTR6149 + 5'LTR2	59	17	9	8 (47%)	90-1000
LTR6149 + LTR6150	59	16	9	7 (44%)	80-900
LTR6149 + Inverse *BARE-1*	52	10	8	2 (20%)	170-500
LTR6149 + PRM3R	52	13	9	4 (31%)	70-750
Direct *BARE-1* + 5'LTR2	59	18	12	6 (33%)	180-1300
Direct *BARE-1* + LTR6150	59	8	6	2 (25%)	270-850
Direct *BARE-1* + Inverse *BARE-1*	52	10	5	5 (50%)	180-480
PRM3F + 5'LTR1	59	11	10	1 (9%)	250-480
PRM3F + 5'LTR2	59	10	10	0 (-)	200-750
PRM3F + LTR6150	59	12	8	4 (33%)	200-800
PRM3F + PRM3R	52	19	14	5 (26%)	170-1000
PRM3(2)F + LTR6150	59	12	10	2 (17%)	210-600
Total	-	197	131	66	70-1300

T_a = annealing temperature.

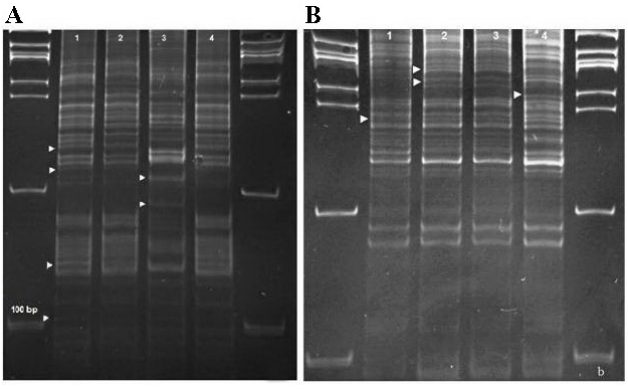

Figure 1. Inter-retrotransposon amplified polymorphism (IRAP) profiles of mature embryo (E, *lane 1*) and calli at different stages (30, 45 and 60 days old, *lanes 2*, *3* and *4*, respectively). M = DNA marker. **A.** IRAP with 3'LTR + 5'LTR2 primers. **B.** IRAP with PRM3F + PRM3R primers. Arrows indicate the polymorphic bands.

Differences in the number and size of bands with different primer combinations (Table 3) could be related matches of the second primer, another retrotransposon, either from the same or a different family in IRAP analyses (Kalendar et al., 1999). Of the total of 197 bands, 131 monomorphic and 66 polymorphic bands were evaluated according to Jaccard's similarity coefficient. IRAP-based similarity indices ranged from 75 to 86% (Table 4).

Table 4. Jaccard's similarity indices (%) between embryo and various callus stages.

	Embryo	30 days old	45 days old	60 days old
Embryo	-	78	75	86
30 days old		-	84	79
45 days old			-	76
60 days old				-

DISCUSSION

Plantlets derived from *in vitro* culture may exhibit somaclonal variation (Larkin and Scowcroft, 1981). Several types of changes, such as chromosome number alterations, are associated with somaclonal variation. Cytogenetic and molecular approaches are generally chosen to detect variations. Random amplified polymorphic DNA (RAPD) is one of the most preferred techniques to evaluate genetic stability among cultured tissues (Yuan et al., 2009; Tyagi et al., 2010). However, somaclonal variation seems to be the result of numerous indels occurring genome-wide accompanied by the activation of retroelements, as a result of stress (Muhammad and Othman, 2005). All known active plant retrotransposons are activated by stresses including wounding, pathogen attack and cell culture. Activation of transposable elements during cell culture has been suggested as a mechanism responsible for somaclonal variation (Wessler, 1996). Therefore, several retrotransposon-based markers, such as IRAP (Muhammad and Othman, 2005; Smykal et al., 2007), have been used to detect changes induced by tissue culture. IRAP requires 2 different primers designed from the LTR sequences of retroelements, and polymorphism is easily detected by the presence or absence of the PCR product. However, annealing temperature, magnesium concentration, quality and amount of *Taq* polymerase and also electrophoresis conditions should be optimized.

A total of 197 bands, 131 monomorphic and 66 polymorphic, were obtained with 14 combinations and evaluated according to Jaccard's similarity coefficient. IRAP-based similarity indices ranged from 75 to 86% (Table 4). The highest similarity value was between embryo and 60-day-old callus (86%). However, the lowest similarity value was between embryo and 45-day-old callus (75%). The analysis of the similarity indices showed that 45-day-old callus is different from other calli and embryo.

BARE-1 movements induced by tissue culture conditions might have occurred during dedifferentiation. Fras et al. (2007) had earlier reported that some polyploidization events were observed after the 3rd day of callus culture. However, due to high similarity to mature embryo tissue, 60-day-old calli may result from different retrotransposon movements in callus induction and developments in different explants. Variations induced by plant tissue culture conditions, such as wounding of explant during excision, 2,4-D treatment, and synthetic medium may be responses to stress conditions. Madlung and Comai (2004) defined tissue culture as "one of the elicitors of genomic stress". Earlier, McClintock (1984) had predicted that stress can cause whole genomic rearrangements, such as loss of chromosomes or chromosome fragments and

break-fusion cycles facilitated by transposition events. Hirochika (1993) was the first to report that tissue culture induced mobilization of tobacco retroelements *Tto1*, *Tto2*, *Tnt1*. Later, Huang et al. (2009) reported the activation of several *nDaiZ* retrotransposons in rice tissue culture. Some applications, such as wounding (Grandbastien, 1998) and exposure to hormones (Takeda et al., 1999), were thought to be responsible for changes occurring during tissue culture. 2,4-D, one of the most preferred hormones to induce callus, is known to cause a dramatic elevation in cytosine methylation (LoSchiavo et al., 1989). Somaclonal variation was documented to occur frequently in plant tissue cultures (Gozukirmizi et al., 1990; Xu et al., 2004; Peredo et al., 2006). Epigenetic modifications especially methylation alterations rather than genetic changes play an important role in somaclonal variation (Peredo et al., 2006; Li et al., 2007; Temel et al., 2008). Methylated cytosines are not distributed evenly in the plant genome; transposons and retrotransposons reside in heavily methylated regions (Rabinowicz et al., 2005). This could explain the alterations in retrotransposon copy number induced by tissue culture.

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