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Nikita retrotransposon movements in callus cultures of barley (*Hordeum vulgare* L.)

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Abstract

Retrotransposons are dynamic elements of the genome and exist in high percentages in the genome of many organisms. However, the majority of retrotransposons are inactivated during development by different mechanisms such as methylation. Some stress conditions may have a stimulating effect on the activation of retrotransposons. *In vitro* culture conditions can be considered as one of these stress factors due to nutrients, chemicals, physical factors and photoperiods. In this study, *Nikita* retrotransposon polymorphism was investigated on different calli ages (30-, 60- and 90-day-old) of barley, which developed from the same embryo on MS medium supplemented with 3 mg/L 2,4-D. Mature barley embryos (*Hordeum vulgare* L. cv. Zafer-160) were cultured for callus formation and sub-cultured every 30 days. Three experiment sets were constructed to determine the polymorphism between individual calli originated from different embryos in the same culture time. Polymorphism was detected using Inter-Retrotransposon Amplified Polymorphism (IRAP) technique with two different *Nikita* specific forward primers. Three mature embryos were used as control. In total, 20 homomorphic PCR bands were obtained from both reactions in intact embryos. However, some polymorphic bands (~ 550 and 650 bp) were solely observed in calli. Our results showed that tissue culture conditions caused the movement of *Nikita* retrotransposon at different ages of calli that originated from the same embryo and at the same time. We explained that all individuals did not show the same effect. To our knowledge, this is the first report on the employment of *Nikita* based IRAP application in barley in terms of callus development.

Keywords: Barley, IRAP, *Nikita*, Retrotransposon, Tissue culture.
Abbreviations: 2,4-D - 2,4-Dichlorophenoxyacetic acid; IRAP - Inter-Retrotransposon Amplified Polymorphism; LTR- Long Terminal Repeats; MS - Murashige and Skoog.

Introduction

Retrotransposons are a subclass of mobile genetic elements and are abundant components of the DNA in many eukaryotic organisms (Kumar and Bennetzen, 1999; Fedoroff, 2000; Zou et al., 2009). They use an RNA intermediate, also known as copy-paste mechanism, to move to their new chromosomal locations. Therefore, retrotransposons cause genome enlargement (Shirasu et al., 2000; Vitte and Panaud, 2005; Grzebelus, 2006). A high percentage of retrotransposons can encode proteins which are needed for transcription, reverse transcription and integration back into the genome. Retrotransposons, which lack those proteins, use the proteins of other retrotransposons. Because of their transposition mechanism and structure, they are thought to resemble retroviruses (Kalendar et al., 2000; Sabot and Schulman, 2006; Sabot et al., 2006). Retrotransposons have a random distribution in the genome. Their ratio to the total DNA in nucleus is very variable in plant kingdom (Kumar et al., 1997; Kalendar et al., 1999). Their abundance in the genome is generally highly correlated with genome size (Schulman and Kalendar, 2005). Especially in cereals, their ratio to the genome is higher than other plants. Therefore, cereals like maize, wheat and barley are excellent model plants for retrotransposon studies. In barley, the most studied retrotransposon is *BARE-1*, a copia-like retrotransposon (Manninen and Schulman, 1993). Shirasu et al. (2000) demonstrated the presence of gypsy-like retrotransposons (e.g. *BAGY-1*, *BAGY-2* and *Sabrina* families) and hybrid retroelement families called *Sukkula*, *Nikita* and *Stowaway* in barley genome (Rodriguez et al., 2006). *Nikita* was reported as the fourth retrotransposon in barley according to activation order (Leigh et al., 2003). Up to date, *Nikita* has been studied for determination of retrotransposon polymorphism in polyploids (Bento et al., 2008; Bento et al., 2010), genetic variability (Carvalho et al., 2010), comparison of different retrotransposon based marker techniques (Zein et al., 2010) and hybrids (Patel et al., 2011). Active retrotransposons may be accepted as major contributors for genome diversification in plants because of their mutagenic potential during transposition and accumulation in the genome (Wessler et al., 1995; Vicient et al., 1999; Schulman and Kalendar, 2005).

Because retrotransposon insertions are irreversible, they are considered as useful genetic elements in phylogenetic studies. Due to their variation capacity between species, retrotransposons are usually studied for detection of genetic relationship between varieties and related species (Waugh et al., 1997; Baumel et al., 2002; Rodriguez et al., 2006; Alavi-Kia et al., 2008; Saeidi et al., 2008; Belyayev et al., 2010; Smykal et al., 2011). Some marker techniques were developed to determine retrotransposon movements in the genome (Schulman et al., 2004). One of these techniques is Inter-Retrotransposon Amplified Polymorphism (IRAP) which depends on amplification of genomic distance between two LTR-retrotransposons that belong to a subclass of retrotransposons (Kalendar and Schulman, 2006). In this

technique, polymorphism is detected by the presence or absence of the PCR product. The lack of amplification indicates the absence of the retrotransposon at the particular locus (Kalendar et al., 1999; Kalendar et al., 2011). IRAP has been used to investigate genetic relationships between varieties and related species (Vicient et al., 2001; Guo et al., 2006; Pasquali et al., 2007), gene mapping (Manninen et al., 2000) and characterization of somaclonal variation (Muhammad and Othman, 2005; Campbell et al., 2011; Evrensel et al., 2011). Generally, a high percentage of retrotransposons are inactivated by various mechanisms (such as DNA methylation) during plant development (Hirochika et al., 2000). However, they may be activated by different biotic or abiotic stress conditions like wounding, pathogen attack, different drug or chemical applications (Wessler, 1996; Grandbastien, 1998; Ikeda et al., 2001). Bonchev et al. (2010) showed ethyl methanesulfonate (EMS)–induced transcriptional activation of *BARE-1* and *WIS 2-1A* retrotransposons.

Plant tissue culture applications have been known as one of the stress conditions. Somaclonal variations are common mutations in tissue culture and result in genetic and phenotypic variations among clonally propagated plants of a single donor (Larkin and Scowcroft, 1981). They can be caused by different kinds of genetic and epigenetic mechanisms such as chromosome breakage and DNA methylation alterations (Gozukirmizi et al., 1990; Temel et al., 2008). Up to date, many studies have been published on somaclonal variation induced by tissue culture. Cytogenetic abnormalities, sequence changes, DNA methylation variations and transposon movements are found in calli, regenerated plants and their progeny (Hirochika, 1993; Hirochika et al., 1996; Kaeppler et al., 2000; Li et al., 2007; Santos et al., 2008).

In this study, we investigated *Nikita* retrotransposon movements on different ages (30-, 60- and 90- day-old calli) of barley calli, which were obtained from the same embryo and then we compared them with three intact mature embryos. This is the first report on *Nikita* retrotransposon movement induced by ageing in tissue culture.

Results

We used IRAP technique to study the effects of tissue culture time on the movement of retroelement *Nikita* in barley calli. IRAP-PCR was performed with three sets of test samples to determine whether there are any different polymorphisms between calli with the same culture time, which originated from different embryos. Each set consists of 30-, 60-, 90-day-old calli that originated from the same embryo. At the same time, to determine whether there are any natural variations between individual embryos, three randomly selected intact mature embryos were compared with one another, regarding the IRAP-PCR profiles of *Nikita*.

A total of 20 bands were amplified from each embryo with both N-57 and E 2647 primers and all of these bands were homomorphic (Fig 1., lane 1-3; Fig 2., lane 1-3). Besides, 20 homomorphic bands were observed in both 30- and 60-day-old calli (Fig 1., lane 4-9), by IRAP-PCR performed with N-57 primer. These bands were also homomorphic with PCR products of embryos. However, 90-day-old calli showed a different band profile. While first 90-day-old callus (Fig 1., lane 10) had the same band profile with intact embryos and the other calli (30- and 60-day-old); the second and third calli had two different novel polymorphic bands (Fig 1., lane 11, 12). These bands were about 550 and 650 bp long, respectively. We also observed some polymorphic bands longer than 2500 bp, but they did not have enough sharp profile for scoring.

The results of IRAP-PCR performed with E 2647 primer were slightly different from those of IRAP-PCR performed with N-57 primer. Both the first and second calli sets (30-, 60-, and 90-day-old) had the same band profiles with the embryos (Fig 2., lane 4, 5, 7, 8, 10, 11), although the third set showed a significantly different novel band (Fig 2., lane 6, 9, 12). This band was about 550 bp long and was observed at all ages of this calli set.

Discussion

In this study, we investigated *Nikita* retrotransposon movements in barley calli of different ages using IRAP technique. Intact mature embryos were used as control. We also used three sets of test samples to determine if there are any different polymorphisms between calli with the same culture time which originated from different embryos. IRAP-PCR that was performed with N-57 and E 2647 primers resulted in 20 homomorphic bands in embryos. These results showed that there is no natural polymorphism between individual embryos for *Nikita* transposition. Therefore, polymorphisms observed in calli at different ages might be resulted from the different tissue culture durations. Some novel polymorphic bands were observed in the calli of different ages, by IRAP-PCR performed with both N-57 and E 2647 primers (Fig 1 and 2, arrowheads). We suggest that these bands were formed as a result of the effect of tissue culture duration on *Nikita* transposition rather than natural polymorphism, as there was no natural polymorphism detected among the intact embryo of individuals. Further polymorphism, which depends on *Nikita* transposition, was not identical in all callus sets. While the first and second callus sets did not show any polymorphism in IRAP-PCR of E 2647 primer, the third set exhibited a 550 bp novel band in each callus of a different age (Fig 2 lane 6, 9, 12). Similarly, in IRAP-PCR with N-57 primer, all 30- and 60-day-old calli were homomorphic with the embryo although 90-day old calli of the second and third sets had two polymorphic bands (Fig 1., lane 11, 12). These different polymorphism profiles of every individual callus indicated that tissue culture conditions do not have the same effect on each individual callus.

Leigh et al. (2003) reported that *Nikita* is the fourth most active retrotransposon in barley. Our previous study on most active retrotransposon *BARE-1* movement in barley calli showed higher polymorphism rates which range between 25% and 14% at various culture times (Evrensel et al., 2011). These results may show that *Nikita* might be less affected than *BARE-1* by tissue culture conditions. Previously, Alavi-Kia et al. (2008) used IRAP technique with 3 barley retrotransposons (*BARE-1, Sukkula* and *Nikita*) and their combination to detect genetic diversity and phylogenetic relationships in *Crocus* genus. They used N-57 primer that we used for amplification of *Nikita*. Although they obtained successful results with combinations of *Nikita* and others (*BARE-1, Sukkula*), they were not able to detect any PCR product using N-57 primer alone. Kalendar et al. (1999) reported that a high number of amplified fragments revealed by retrotransposon markers could be due to their high copy number in the genome. Therefore, Alavi-Kia et al. (2008) suggested that *Nikita* may not have an important role in genome construction of *Crocus* genus. However, we could obtain scorable band profiles with both N-57 and E 2647 primers. This may indicate that *Nikita* have a possible role

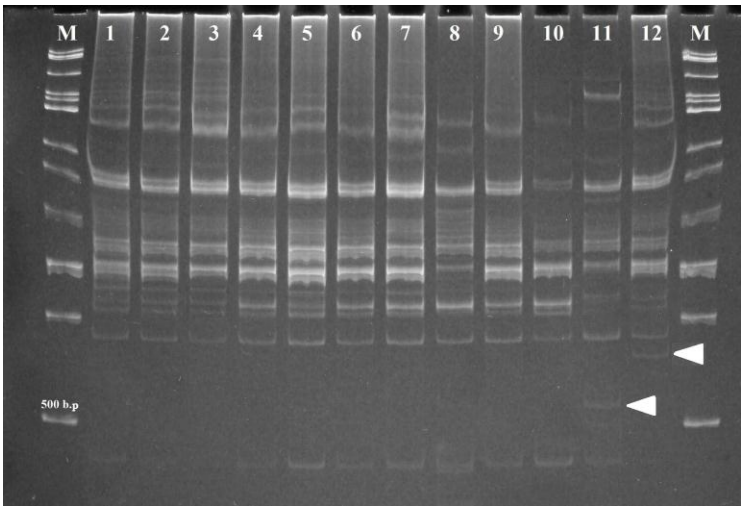

Fig 1. IRAP result obtained from N-57 primer reaction. Lanes 1-3; non-cultured mature embryos; Lanes 4, 5, 6 are from 30 day-old calli originated from different embryos; Lanes 7, 8, 9 from 60 day-old calli originated from different embryos; Lanes 10, 11, 12 are from 90 day-old calli originated from different embryos (Polymorphic bands were shown with arrowheads).

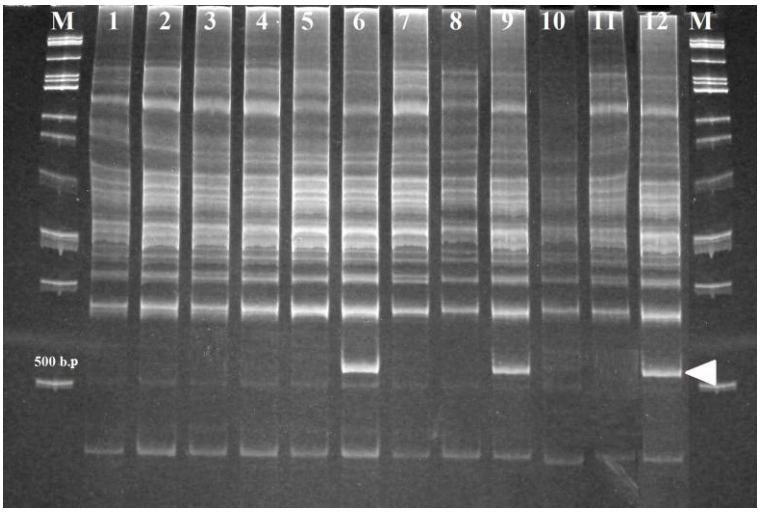

Fig 2. IRAP result obtained from E 2647 primer reaction. Lanes 1-3 from non-cultured mature embryos; lanes 4, 5, 6 from 30 day-old calli originated from different embryos; Lanes 7, 8, 9 from 60 day-old calli originated from different embryos; Lanes 10, 11, 12 from 90 day-old calli originated from different embryos (Polymorphic bands were shown with arrowhead).

like *BARE-1* and *Sukkula* in the genome construction of barley due to its high copy number. Campbell et al. (2010) used IRAP technique to detect somaclonal variation based on *BARE-1* retrotransposon polymorphism at 147 primary regenerants in barley tissue culture. They reported that IRAP is a useful technique for detection of mutations which are induced by tissue culture conditions. Our results also showed that IRAP is a useful method to determine somaclonal variation caused by *Nikita* transposition in barley callus culture.

All the IRAP-PCR results obtained in this study indicate that tissue culture conditions may induce 2/20 bands=0.1 transposition of *Nikita* retrotransposon. However, this transposition ratio was related to the calli age and showed differences in each individual callus.

Materials and methods

Callus Culture

Barley (*Hordeum vulgare* cv. Zafer-160) seeds were surface-sterilized with commercial bleach for 20 min and rinsed with sterile dH_2O 3 times for 10 min. After sterilization, mature embryos were removed from seeds and dipped in absolute ethanol for 30 sec. Ethanol was removed and seeds were rinsed with sterile dH_2O 3 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 2,4-D (Sigma, D7299). Each embryo was given a number and incubated at 25 ± 2°C in complete darkness for 30 days. At the end of the incubation time, each callus was cut into two pieces. One of the pieces was used for genomic DNA isolation and the other was sub-cultured at the same conditions for 30 days. These sub-culture and DNA isolation steps were also performed at days 60 and 90. Genomic DNA was also isolated from non-cultured mature embryos to use as a control in PCR.

Genomic DNA Isolation

Genomic DNA was isolated from non-cultured embryo and 30, 60, 90-day-old calli according to Rogers and Bendich (1985). Each calli set of different ages originated from one

embryo. Quality of DNAs was controlled with 1% agarose gel and quantity of DNAs was measured by spectrophotometer.

Inter-Retrotransposon Amplified Polymorphism (IRAP) PCR

The IRAP was performed with two different forward primers designed for LTRs-sequences of *Nikita* retrotransposon (N-57: 5'CGCATTTGTTCAAGCCTAAACC 3' and E2647: 5'ACCCCTCTAGGCGACATCC3'). Because of the sequence similarity between left and right LTR which flank *Nikita* retrotransposon, only one primer can be used for IRAP-PCR of *Nikita* as both forward and reverse. Therefore, one of these primers was used for each PCR. Amplification reactions were carried out in 20 µl reaction volume containing 9.9 µl nuclease-free dH_2O, 2.0 µl 10X buffer (1X), 2.0 µl 25 mM $MgCl_2$ (2.5 mM), 2 µl 10 mM (2.5 mM each) dNTP mixture (1 mM), 1.6 µl primer (8 pmol, 0.8 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 amplification conditions were set up as one initial denaturation step at 94°C for 2 min followed by 30 cycles at 94ºC for 30 s, at 52°C for N-57 and 53°C for E 2647 for 30 s and at 72°C for 3 min. The reactions were completed with a final extension step at 72°C for 7 min.

Evaluation of PCR products

Polyacrylamide gel electrophoresis was employed to separate PCR products. Ten-µl aliquots of IRAP-PCR products were 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 8% nondenaturing polyacrylamide (29:1 Acrylamide:Bis) gels at 200V for 6 h in 1X TBE buffer (90 mM Tris-borate and 2 mM EDTA, pH 8.0). A molecular weight marker (GeneRuler™ 1 kb DNA Ladder, SM0312, Fermentas) was also loaded to determine the sizes of amplicons. Gels were stained in 1X TBE buffer containing 0.5 µg/ml ethidium bromide for 15 minutes. After staining, gels were rinsed with distilled water and photographed on a UV transilluminator and scored visually.

Conclusion

In this study, *Nikita* retrotransposon integration events in mature embryos, and 30-, 60 and 90-day-old calli were investigated. Our results showed that some retrotransposition events occur during callus induction and callus development. However, sequence characterization of the regions which exhibit polymorphism is crucial for the exploration of callus development and retrotransposition. To mention somaclonal variation, one should analyze not only calli but also regenerated plantlets and/or plants. Regeneration studies are still being carried out.

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