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Detection of *in vitro* culture-induced instability through inter-simple sequence repeat analysis

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Abstract This paper reports on investigations focused on trinucleotide and tetranucleotide repeats in cauliflower calli (*Brassica oleracea* var. *botrytis* L.) and carried out to determine their utility in the detection of genetic variations induced by tissue culture. Out of 224 calli 6 exhibited original patterns; in one of these, PCR patterns differed at four polymorphic loci. The observed tetranucleotide-repeat classes were polymorphic, whereas fingerprinting patterns were stable with $(CAG)_5$. The most frequent polymorphic and useful primer for detecting genetic variation appeared to be $(CAA)_5$. We also characterised an Inter-Simple Sequence Repeat (ISSR) marker homologous to a gene involved in cellular proliferation, and modifications of this gene on callogenesis and/or differentiation are examined.

Keywords Calli · Cauliflower · Genetic instability · Microsatellite primers · Somaclonal variation

Introduction

Reliable assays to assess the genetic stability of a genotype throughout tissue culture are highly desirable in micropropagation and genetic engineering. Various methods have been used to characterise somaclonal variation or to distinguish among mutation-derived cultivars. Isozymes and cytological analysis of somaclones have been used in many plant species (for review, see Jain et al. 1997), but these approaches are time-consuming, tedious and often affected by physiological and environmental factors. Thus, molecular tools appear to be more reliable for the assessment of variation. Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) have been applied to study plant-genome diversity, stability and evolution. These techniques, however, do not target rapidly evolving sequences and because a mutational event affects only a very small proportion of the genome, the detection of such variation will require a high number of enzyme-probes or primers.

Microsatellites or Simple Sequence Repeats (SSRs) consist of short tandem repeats based on two to five base-pair motifs; they are highly informative and distributed throughout eukaryotic genomes (Tautz and Renz 1984; Morgante and Olivieri 1993). The detected polymorphism is based on the number of di-, tri- or tetra-nucleotide repeats. In addition, flanking regions must be known to generate polymerase chain reaction (PCR) primers. On the other hand, with Inter-Simple Sequence Repeats (ISSRs) no prior information on the sequence is required, and reproducible polymorphisms are produced in high number.

Microsatellite primers have been used for assessing genetic diversity in corn (Kantety et al. 1995) and wheat (Nagaoka and Ogihara 1997) and for identifying cultivars in *Chrysanthemum* and *Citrus* pooled samples (Wolff et al. 1995; Fang and Roose 1997) and individual plants of oilseed rape cultivars (Charters et al. 1996) and flax anther culture-derived plants of microspore origin (Chen et al. 1998). This paper reports on the use of microsatellite primers to assess genetic stability in tissue culture. It also highlights the interest given to detecting altered bands as they provide valuable information on where genomic events have occurred.

Materials and methods

Plant materials

Seed samples of cauliflowers. (cvs Meurz, Jakez, H524 and double haploid (DH) lines 2, 3, 5 and 6) were obtained from the Organisation Bretonne de Sélection (OBS, Plougoulm, France).

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Tissue cultures

Seeds were soaked in tap water (50 ml water) containing Tween 20 (2 drops) for 6 min, surface-sterilised in hydrogen peroxide (11 vol) for 15 min, washed with sterile distilled water and placed in 7% (w/v) calcium hypochlorite solution for 20 min. The, seeds were then rinsed three times in sterile distilled water and aseptically germinated on basal MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose, solidified with 0.8 % (w/v) Sobigel agar at pH 5.7. Three-week-old hypocotyls were excised into 5-mm-long sections and used as explants for the in vitro experiments. Basal media were autoclaved (120°C , 20 min), and plant growth factors were filter–sterilised (Millipore HAWP, 0.22-µm pore size) and added after autoclaving. The explants were placed for 3 weeks on a basal MS medium containing thidiazuron (TDZ), dicamba (D), 6-benzyl-amino purine (BAP) and α-naphthalene-acetic acid (NAA) in various concentrations: dicamba alone (10 µg. l^{-1}); dicamba (5 µg. l^{-1}) associated with thidiazuron (10 µg. l^{-1}); BAP (4 mg l^{-1}) with NAA (0.75 mgl^{-1}). Experiments were conducted at 25±1°C under a 16-h (light) photoperiod of cool white fluorescent light (25 µE m^{-2} s^{-1}). The calli that formed on hypocotyl explants were collected and named CJ, CH, CM, CD for Jakez, H524, Meurz and DH calli, respectively. For some experiments, half of calli were subcultured on a fresh medium similar to the first one. Leaves from the mother plant and calli were stored individually at –80°C until DNA extraction.

DNA extraction

DNA was isolated from each leaf or callus according to a CTAB protocol. Samples were ground in extraction buffer [100 m*M* TRIS pH 8.0, 1.4 m*M* NaCl, 20 m*M* EDTA pH 8.0, 0.2 % (w/v) β-mercapto-ethanol, 2% (w/v) CTAB (cetyl trimethyl ammonium bromide], and heated at 60°C for 30 min. DNA was extracted with one volume of chloroform/isoamyl–alcohol (24/1) and precipitated in the presence of isopropanol [40% (v/v) final concentration]. The DNA pellet was spun down by centrifugation at 12000 *g* for 5 min, washed with 5 m*M* ammonium acetate and 70% ethanol, then dried and re-dissolved in 100 µl of TE (10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA pH 8.0). After the addition of 1 µl of RNAse (10 mg ml^{-1}) the quality of the DNA preparations was checked by agarose gel electrophoresis, and the DNA concentrations were determined on a fluorometer (Hoefer TKO 100) using bisbenzimide (Hoechst dye 33258) as the fluorescent dye.

Amplification conditions of ISSRs

Amplification reactions were performed in 25-µl volumes containing 75 m*M* TRIS-HCl pH 9.0, 20 m*M* $(NH_4)_2SO_4$, 0.001% (w/v) Tween 20, 2.5 m*M* $MgCl_2$, dATP, dCTP, dGTP and dTTP (Eurogentec) in equal amounts (200 µm each), 0.1 n*M* primer, 15 ng of genomic DNA and 1.25 U *Taq* DNA polymerase (Goldstar 'red', Eurogentec). PCR amplifications were conducted in a Perkin Elmer GeneAmp PCR 2400 Thermal Cycler programmed for 27 rounds of the following amplification cycle: 94°C for 1 min; 42°C (GATAGATAGATAGATA), 54°C (CAACAACAACAACAA), 56°C (GACAGACAGACAGACA) or 62°C (CAGCAGCAGCAGCAG) for 1 min, 72°C for 4 min. An initial denaturation step at 94°C for 1 min and a final extension at 72°C for 7 min were added. PCR products were separated on 2% agarose gels buffered with 1×TAE (40 m*M* TRIS acetate, 1 m*M* EDTA pH 8.0) and stained with ethidium bromide. Gel images were acquired and analysed with Gel Doc 1000 (Bio-Rad).

Cloning and sequencing

To convert bands from an ISSR gel into single locus markers we first excised them from the dried gel. The DNAs were eluted from the gel slices in 50 µl of TE, purified by centrifugation at 10000 rpm for 6 min after the addition of 1 vol chloroform/isoamyl-alcohol. DNA was precipitated by adding 1 vol 3 *M* ammonium acetate and 0.020 vol 20 mg ml^{-1} glycogen, incubated at –20°C 2 h, then centrifuged at 12000 rpm for 10 min. The pellet DNA was washed once in 70% ethanol, air-dried, then re-suspended in 10 µl TE; 1 µl of this DNA solution was used as a template for PCR amplification under the conditions previously described. These re-amplification products were sub-cloned into a pGEM-T Easy Vector (Promega). E. coli DH5α strain (*supE*44 Δlac U169 (F80 lacZΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) (Sambrook et al. 1989) was used for the transformation experiments and for the propagation of plasmids. Plasmid DNA was extracted from the transformed colonies according to Sambrook et al. (1989) and re-amplified by specific T7 and SP6 primers (Promega). For optimum results, the PCR products were purified and concentrated by ultrafiltration through anisotropic membranes (Centricon YM-100 columns, Millipore). The amount of purified DNA was quantified by measuring absorbance at 260 nm. Cycle sequencing was per-

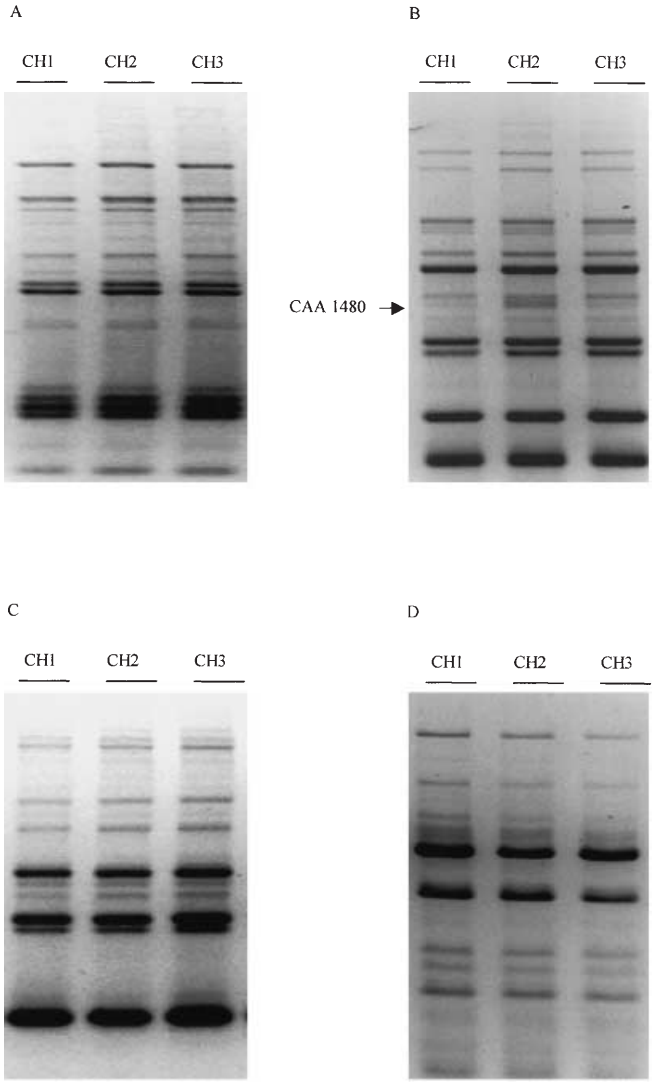

Fig. 1A–D PCR analysis of three brother cauliflower (Brassica oleracea var. botrytis L.) calli (CH1, -2 and –3) using primers. Calli were generated from the same mother plant (genotype H524). Amplification products from the ISSR reaction were obtained using primers $(CAG)_5$ (**A**), $(CAA)_5$ (**B**), $(GACA)_4$ (**C**) and $(GATA)_4$ (**D**). The *arrow* indicates a polymorphic fragment; designated CAA 1480. Polymorphic markers were labelled as follows: first the name followed by the molecular size of the marker in script

formed in a GeneAmp PCR system 9600 (PE Applied Biosystems) using 10 ng PCR product DNA, 3.2 pmol primer, 8 µl Terminator Ready Reaction Mix and deionised water to a total volume of 20 µl. Optimised thermal cycling conditions consisted of 25 cycles as follows: 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Once the DNA sequencing reactions had been completed, the excess of BigDye terminators was removed by purification of extension products on Centi-Sep spin columns (Princeton Separations). PCR product electrophoresis was performed on an ABI Prism 310 Genetic Analyser.

Results

Identification of informative ISSR markers

DNA analyses were carried out on genetic material from various cauliflower calli. ISSR-PCR tests were performed using the microsatellite primers $(CAG)_5$, $(CAA)_5$, $(GACA)_4$ and $(GATA)_4$ for they had been proved useful in previous studies on different taxa. These primers of variable length, 15 or 16 nucleotides, had a G+C content within 25% and 66.6 %. All of them amplified scorable fragments between either 250 and, 2000 bp or 250 and 3000 bp, depending on the primer used. Obvious variations between cauliflower species were observed, but they are not reported here since they did not match up to the aim of the present study. Fingerprint patterns were obtained for each mother plant and used to define 'normal' and 'variant' calli. Two hundred and twenty four calli derived from seven cultivars and three in vitro culture media were tested; four CH2, CJ2, CM4 and CM5, were polymorphic as compared to the mother plant, whereas two others, *i.e.* CD521and CD522, were polymorphic to both the mother plant and callus. These calli had been respectively obtained on dicamba medium for CH, on dicamba and thidiazuron for CJ and CM, and on BAP and NAA for CD. Variations in fingerprinting patterns resulted in the occurence of one 1480-bp band (CAA_5) for the first variant (CH2, Fig. 1) and of two 2000-bp fragments (CAA_5 and $GATA_4$) along with the deletion of two 300-bp and 800-bp fragments (CAA_5 and $GACA_4$) for the second one (CJ2, Fig. 2). On the other hand, the third variant

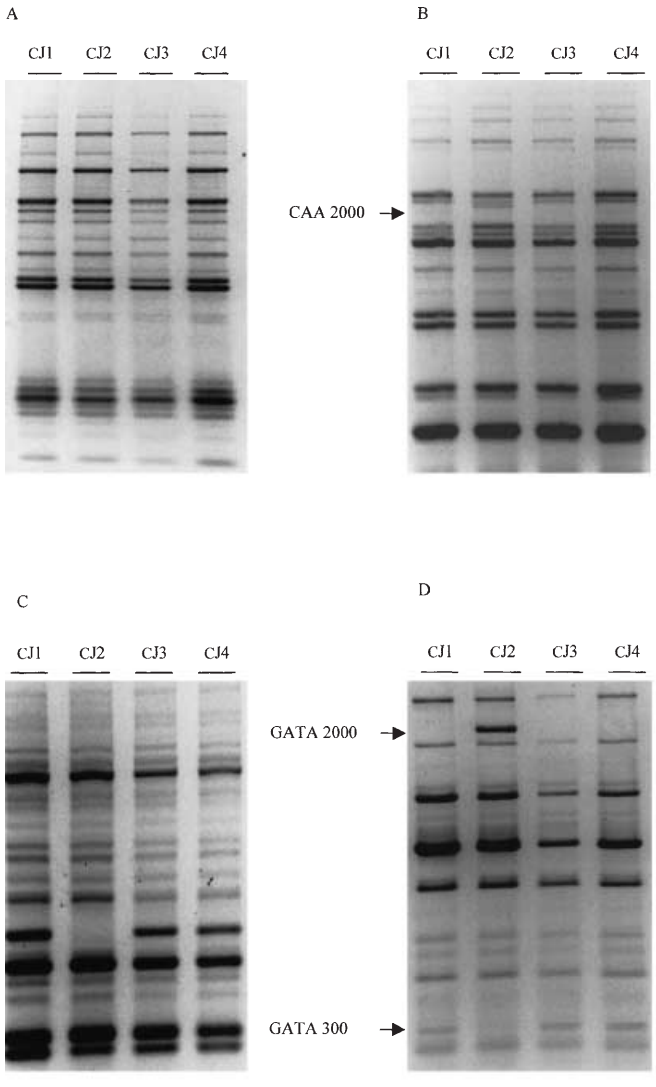

Fig. 2A–D Banding profiles of four cauliflower calli (CJ1–4) generated from the same mother plant (genotype Jakez). Amplification products from the ISSR reaction were obtained using primers $(CAG)_5$ (**A**), $(CAA)_5$ (**B**), $(GACA)_4$ (**C**) and $(GATA)_4$ (**D**). Polymorphic fragments, CAA2000, GACA 800, GATA 800 and GATA 2000 are shown (*arrows*)

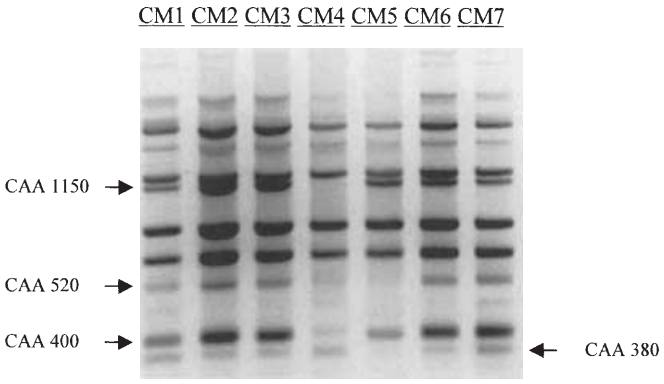

Fig. 3 Amplification products from the ISSR reaction obtained from genotype Meurz using the primer $(CAA)_5$. *Arrows* indicate polymorphic markers

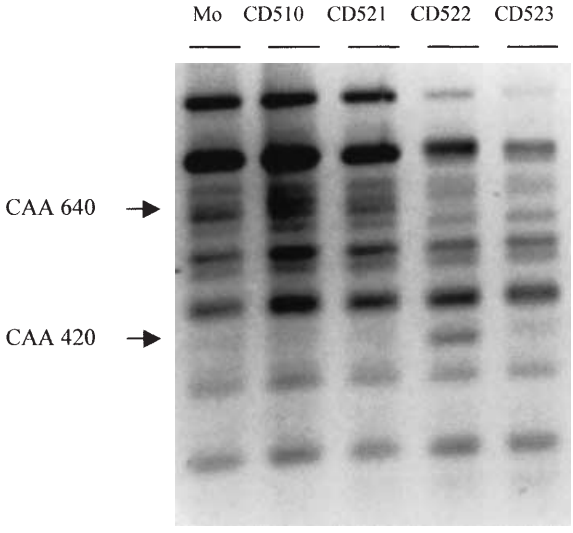

Fig. 4 Fingerprint patterns of a family tree. Markers were generated with $(CAA)_5$. *Mo* Mother plant, *CD510* callus of first generation, *CD521–522* and *–523*: calli descended from CD510

(CM4) was characterised by the loss of three markers of 1150, 520 bp and 400 bp (CAA_5), respectively, whereas the last one (CM5) lost two fragments of 520 and 380 bp (Fig. 3). Changes in fingerprint patterns at the two loci CAA 640 and CAA 420 characterised the somaclonal variant CD522, whereas those at the locus CAA 640 were typical of CD523 (Fig. 4). No differences were observed when $(CAG)_5$ was used. $(CAA)_5$ enabled the detection of polymorphisms in six calli, whereas $(GACA)_4$ and $(GATA)_4$ confirmed the genetic variation of the same callus (Fig. 2). The polymorphism between calli was clear and, as shown in Table 1, 11 polymorphic markers were produced. The extent of variations observed in cvs. Jakez and Meurz was higher than that obtained within H524 (Leroy et al. 1997) or DH calli.

Polymorphic patterns upon ISSR analysis appeared not only at the first callus phase but also after a subculture through the analysis of a DH family (Fig. 4). Two second-generation calli (CD522 and CD523) were indeed different from their brother callus (CD521) and from Mo and CD510, their mother plant and callus, respectively.

Genomic instability was evaluated for each primer using the Polymorphism Index Content (PIC). PIC values give an account of the bands generated from normal calli and of those altered in somaclonal variant DNA. The data given in Table 1 suggest an initiation of genomic instability at an early stage of calli formation. Consequently, such alterations have a higher rate of occurrence during cellular proliferation this will result in a greater number of genomic lesions in calli at a later stage.

DNA sequencing of the amplified fragments

Previous shotgun-cloning and sequencing of ISSR PCR products amplified with cauliflower microsatellite primers confirmed that all fragments contained simple SSRs at their ends (data not shown). Since the observed variations may result in a change in the microsatellite-repetition number or in the sequence between SSRs, we expanded our DNA sequencing to include five variant loci. We performed BLAST searches for nucleotide and amino acid similarity to determine whether the unstable ISSRs were associated or not with conserved gene regions. BLAST searches were based on the full-length sequence: among five cloned loci, only one (GATA300) showed any similarity to any GenBank sequences. This particular sequence (Fig. 5) contained a stretch of high sequence similarity (78% identity, 80% similarity at the amino acid level) to a predicted serine-threonine kinase (*Arabidopsis thaliana*, Y11930), itself homologous with human and mouse *ttk* genes.

This sequence was also found to be very interesting due to its characterisation by simple perfect SSRs at its ends (Weber and May 1989): indeed, GATA showed at least four repetitions, and the 3′-end of this sequence was adjacent to a block of imperfect tandem repeats of a $(GT)_{12}$ sequence. One should note that, up to now, accurate characterisation of compound repeats was not available, and their potential informativeness has not yet been established.

Of the 11 altered PCR products 4 represented new specific bands lacking a visible normal tissue counterpart; this observation suggests that, in these cases, the genomic event had occurred either between the two primers or at the fixation site of one primer. We also analysed the ISSR-PCR data to estimate the total number of genomic alterations and events that had occurred in the variant calli studied. The number of alterations can be calculated as follows: [number of alterations/size of genome screened]×genome size. As 11 somaclonal polymorphisms have been detected in 15 of the 149 ISSR bands scored from 224 cauliflower calli, we estimated that the occurrence of individual genomic events in CJ2 callus must have been equal to 52000.

Table 1 Polymorphic markers from three cauliflower calli. This table shows the polymorphic index (PIC) and the sizes of the polymorphic markers obtained on different media, generated by different primers and revealed on agarose gels. Presence (+) or absence (–) of markers are indicated for plant, normal calli (NC), and variant calli (VC)

Primer	Polymorphic markers	Genotype	PI % (pol mk/∑mk)	Characteristics		
				Plant/NC	VC	Mediuma
CAA	CAA 2000	Jakez	5.88 (1/17)	–	+	D+TDZ
	CAA 1480	H_{524}	5.55 (1/18)	–	+	D
	CAA 1150	Meurz	6.66 (1/15)	+	–	D+TDZ
	CAA 520	Meurz	20.0 (3/15)	+	–	D+TDZ
	CAA 400	Meurz	–	+	–	D+TDZ
	CAA 380	Meurz	–	+	–	D+TDZ
	CAA 640	DH_5	11.8 (2/17)	+	–	B+NAA
	CAA 420	DH_5	–	–	+/–	B+NAA
GACA	GACA 800	Jakez	4.16 (1/24)	+	–	D+TDZ
GATA	GATA 300	Jakez	11.8 (2/17)	+	–	D+TDZ
	GATA 2000	Jakez	–	–	+	D+TDZ

[a] D, Dicamba; D+TDZ, dicamba+thidiazuron; B+NAA, 6-benzyl-amino purine; + α-naphthalene-acetic acid

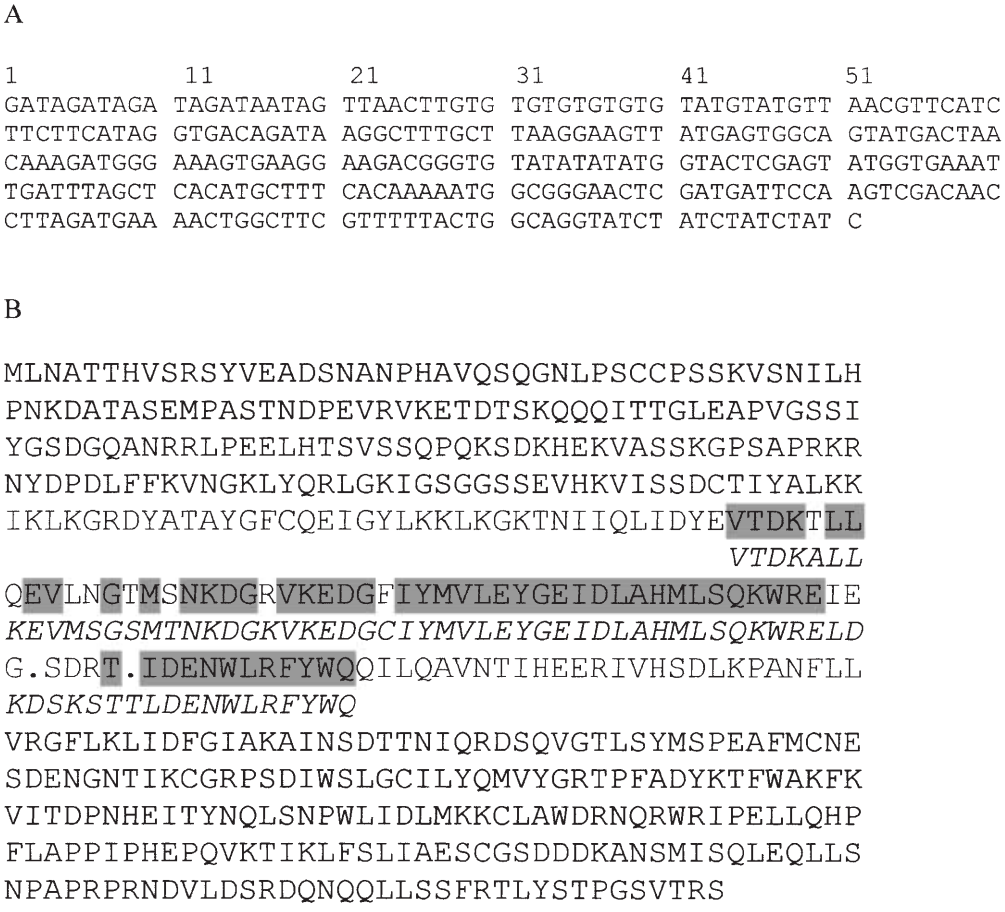

Fig. 5A, B GATA sequence missed in one variant callus and homologous with an *A. thaliana* protein kinase. **A** Nucleotide sequence of the polymorphic marker. **B** Sequence alignment between deduced amino acid sequences of the *B. oleracea* predicted gene *bok1* and the *A. thaliana* deduced protein kinase

Discussion

Over the last 15 years fingerprinting-based investigations have focused on displaying genetic variations in plants under various culture conditions. Indeed using isozymes and RFLPs, Sabir et al. (1992) reported on variations in *in vitro*-regenerated beet plants, whereas Munthali et al. (1992) employed RAPDs to investigate the same material. Through a similar approach, Dan and Stephens (1997) generated RAPD markers in two *Asparagus* somaclonal lines resistant to *Fusarium oxysporum* f. sp. *asparagi* and distinguished the resistant somaclonal lines from the parental cultivar. With a minisatellite probe Dallas (1988) characterised rice somaclones issued

from protoplasts. Nelke et al. (1993) identified a regenerative somaclonal variant of red clover by using DNA fingerprints generated by Jeffrey's probe 33.6. Poulsen et al. (1993) revealed somaclonal variation at the DNA level using $(GATA)_4$ as a probe in *Brassica napus*, but only two bands exhibited very minor variations. These authors along with Karp (1991) have proposed that changes in methylation could be responsible for heritable variation. However, 17 DNA extractions from somaclonal variants were used in rice for the detection of genetic variation by hybridisation with microsatellite probes. None of the tested probes, *i.e.* $(GATA)_4$, $(CAC)_5$ and $(TG)_{10}$, revealed polymorphism (Chowdari et al. 1998). Similar results have been obtained by Schmidt et al. (1993) in *Beta vulgaris*. Studies of Vosman et al. (1992) in tomato cv. Moneymaker with $(GATA)_4$-probe showed polymorphism neither in the 95 calli-lines nor in 45 R1 plants derived from tissue culture regenerants.

Wolff et al. (1995) used RAPDs and inter-SSR PCR for detecting polymorphism in DNA from material kept either in tissue culture or vegetatively propagated. They found no RAPD or SSR-PCR differences between phenotypically different members of a family, which led them to suppose that they had failed in showing polymorphism because the part of the genome studied was too reduced or the number of primers used too low.

The results obtained in our study not only suggest that genetic variations may take place during the callus step but also illustrate how the impact of culture conditions on fingerprint patterns can be assessed through the use of microsatellite primers. Such variations may, indeed, result from changes in either the hormonal composition of the media used or an undifferentiated cell step. Somaclonal variation was observed by Jain et al. (1989) in *Brassica juncea* cotyledonary calli; these variants had been induced on MS medium supplemented with high cytokinin (kinetin or zeatin) and low indole-acetic acid concentrations. Another proposed explanation is that the plants regenerated from unorganised callus vary more than those from organised callus, whereas no or very little variation occurs when plants are regenerated directly without an intermediate callus phase (Larkin et al. 1995). In a previous study based on an ISSR-PCR approach, we did not observe any substantial genetic differences between plants regenerated *via* somatic embryogenesis (Leroy et al. 2000). It therefore appears that the variations are probably 'cancelled' during the embryogenic phase (to be confirmed).

Several genetic mechanisms, including gene amplification by processes such as replication slippage, rolling circle amplification, unequal exchange and mutation by base substitutions, can affect the number of repeating units in tandem arrays. Fisher et al. (1996) observed the presence of additional internal microsatellites for some of the ISSR markers. Such an observation would imply a clustering of microsatellites in some genomic regions. Evidence is provided by the sequence displayed above (Fig. 5A) along with other unpublished data. From in vitro studies, it has been suggested that strand slippage during DNA replication may be the major cause of the length polymorphism observed between populations. This process results in changing length by the addition or deletion of a few repeats at a time. There is apparently a relation between replication slippage of microsatellite sequences and defective DNA repair, since mutations in DNA repair genes can cause tract instability when human genetic diseases are associated with variations in the length of triplet repeats. The mechanism of expansion is still unclear, and many hypotheses involve abnormal replication or repair processes on a slipped strand structure within the triplet region.

In the study reported here, genomic instability in cauliflower calli produced up to 50000 genomic events per cell. In addition, ISSR technology detected significant levels of DNA polymorphism at the first callus phase; this result proves that this approach constitutes a fast and efficient technique in the assessment of variations obtained through tissue culture. The alteration observed in the fingerprinting pattern with the loss of a marker corresponding to a part of a gene involved in the regulation of cell proliferation would be the consequence of microsatellite instability at the primer fixative site, or of modification(s) either at the poly (GT) SSR or between the microsatellites. However, all these results highlight that the use of microsatellites or ISSRs is more complex than previously thought. The selective conditions present in the callus environment differ from those found in the environment of 'normal' cells. As a result, mutations in growth-controlling genes may occur at the same rate in variant cells as in 'normal' cells, but the selective advantage provided by such a mutation in a variant-cell environment could give rise to clonal expansions and allow the mutated cell to overtake its sister cells. If the same mutation occurred in non-callogenesis cells in the absence of clonal expansions, it would be undetectable. In our case, the inactivation (or invalidation) of the protein kinase gene would generate a specific phenotype, unobserved because of the sacrifice of the calli. Northern blot analysis revealed that the *ttk* gene was expressed in tissues with a large number of rapidly proliferating cells, such as the testis and thymus. The plant homologue *ttk* gene can indeed be characterised by at least three microsatellites. As defined by Hardie (1999), plant serine/threonine-kinase accepts information from receptors that sense environmental conditions, phytohormones and other external factors and converts it into appropriate outputs such as changes in metabolism, gene expression and cell growth and division. The discovery of a mutation in this gene could be the starting point of further investigations aimed at a better understanding of cell proliferation and differentiation. Tracts of polyglutamine, coded by $(CAG)_5$ or $(CAA)_5$, have been found in transcription factors and are known to play a role in normal protein function; this has been shown by their ability to activate transcription when present in DNA-binding proteins. As the instabilities of cauliflower calli are likely to be genetically based and highlighted by $(CAA)_5$, they will help us to distinguish variants from 'normal' cells. By analogy with tumorigenesis, calli or plants with abnormal ISSR

patterns can now be said to have IMSI (inter-microsatellite instability). Because instabilities reflect defects in the cellular process that maintains the integrity of the genome, they can be expected to generate sensitivities to particular chemicals agents and/or growth regulators.

Conclusions

The data reported here highlight that genomic instability can be detected at a very early stage. Three of the four microsatellite primers tested proved to be efficient in revealing clear changes in banding profiles. ISSRs require low DNA quantities, generate a high number of markers and are highly reproducible, which make them a candidate of choice in the study of plant genome stability/instability.

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