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Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs

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Abstract To study the somatic embryogenesis of *Brassica oleracea* var. *botrytis* L., hypocotyls were placed on Murashige and Skoog's medium (1962) with 1 mg.l^{-1} of 2,4-dichlorophenoxyacetic acid and 1 mg.l^{-1} of kinetin to induce callogenesis. After transfer of the calli to the maturation medium, somatic embryos appeared. They developed into plantlets and the potential of regeneration of the calli was maintained for more than 8 months. Thirty-five plantlets were produced after 2 months of culture, then transplanted into soil. Inter-simple sequence repeat markers generated by trinucleotidic and tetranucleotidic primers were tested for their ability to characterise genomic variations in the obtained plants. The absence of polymorphism between different regenerants from the same cultivar indicates the conformity of the regeneration protocol.

Key words Somatic embryogenesis · Inter-simple sequence repeat · Stability · Cauliflower · Plant regeneration

Abbreviations *2,4-D* · 2,4-Dichlorophenoxyacetic acid · *Kin* Kinetin · *MS* Murashige and Skoog (1962) basal medium · *ISSRs* Inter-simple sequence repeats · *SEIM* Somatic embryo induction medium · *SEMM* Somatic embryo maturation medium

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Introduction

Regeneration of whole plants from cultured tissues or cells is a prerequisite for successful applications of *in vitro* techniques of gene transfer, mass propagation and somaclonal variation studies. An adventitious regeneration process via somatic embryogenesis is highly desirable since it allows high rates of multiplication and results in true-to-type plants. Somatic embryogenesis is, indeed, characterised by the formation of an external bipolar structure from plant tissues without connection to the plant vascular system; moreover, this structure bears root and shoot poles. This development can be the single noticeable event during this process. Its use has been emphasised as an efficient method for a fast *in vitro* multiplication of plants as well as a tool for crop improvement. Embryogenic cell-lines retain their regenerating potential for a long period in culture and often give rise to a uniform and normal population (Vasil 1982). Thus, the most promising regeneration system to integrate genetic transformation, for example with particle bombardment, is indirect somatic embryogenesis.

Considerable advances towards the development of embryogenic cultures have been made for a lot of *Brassica* species. In the most documented examples, the explants used consisted of microspores and protoplasts (Chuong and Beversdort 1985; Keller et al. 1986; Zhao et al. 1996). Cauliflower plants have been regenerated from complex explants via *in vitro* organogenesis from roots (Lazzeri and Dunwell 1984; Horeau et al. 1988), cotyledons (Murata and Orton 1987; Horeau et al. 1988; Jain et al. 1988; Narasimhulu and Chopra 1988), peduncles (Christey and Earle 1991), protoplasts (Delpierre and Boccon-Gibod 1992), but to our knowledge, the only report about somatic embryogenesis was published by Pareek and Chandra (1978). These authors did not mention plant regeneration; in addition, the structures drawn in their report might have been not only young somatic embryos, but also

trumpet-like structures. The present study reports on how cauliflower plants were obtained for the first time through somatic embryos; it also highlights the stability of regenerants from a molecular study carried out by inter-simple sequence repeat PCR.

Materials and methods

Plant material

Seeds of *Brassica oleracea* var. *botrytis* L. H524, a hybrid, were soaked in Tween-enriched tap water (2 drops/50 ml water) for 6 min, surface-sterilised in hydrogen peroxide 11 vols for 15 min, washed with sterile distilled water and then placed in 7% (w/v) calcium hypochlorite solution for 20 min. Then, they were rinsed three times in sterile distilled water and aseptically germinated on MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose, solidified with 0.8% (w/v) Sobigel agar, at pH 5.7. One- to 3-week-old hypocotyls were excised into sections of 10 mm in length and used as explants for all the *in vitro* experiments.

Culture conditions

The explants were placed onto an MS medium containing 0.1, 0.5 or 1 $mg.l^{-1}$ of 2,4-D alone or in combination with Kin at 0.1, 0.5 or 1 $mg.l^{-1}$ for 3 weeks. Every 3 weeks the calli formed on hypocotyl explants were subcultured on a medium containing 2,4-D and Kin at 0.1 or 1 $mg.l^{-1}$. The experiments were conducted at 25 ± 1 °C under a 16-h photoperiod regime of cool white fluorescent light ($35\ \mu E.m^{-2}.s^{-1}$). Basal media were autoclaved (120 °C/20 min), and all growth factors were filter-sterilised (Millipore HAWP 0.22 μm) and added after autoclaving. The embryos were hand-picked under the microscope and divided into five classes with respect to their stage and size: (1) globular, less than or equal to 80 mm; (2) heart-like, range 80–200 mm; (3) small torpedoes, range 200–350 mm; (4) straight torpedoes, range 350–500 mm; (5) bent torpedoes, above 500 mm. Hypocotylary embryos were transferred onto solid MS medium. To select the best medium, statistical analysis was made using a χ^2 criterion.

Acclimatisation and transfer of plantlets to soil

Five-centimetre plantlets were removed from agar medium and transferred to compost. Rooted plants were gradually acclimatised in a controlled growth chamber under a 14-h photoperiod and thermoperiod (light and $T = 22 \pm 1$ °C with hygrometry = 75%; dark and $T = 18 \pm 1$ °C with hygrometry = 80%). One month later, they were transferred to the greenhouse.

DNA extraction and ISSR amplification

DNA was extracted from young leaf tissues (100 mg) and calli (25–50 mg) following a CTAB (cetyltrimethylammonium bromide) protocol (Saghai-Maroof et al. 1984).

Four primers were tested for ISSR amplification in the cauliflower genome. A single primer was used in each PCR reaction, which was carried out in a total volume of 25 μl containing 15 ng of genomic DNA (1 μl), 2.5 μl of 10 × buffer (Eurogentec), 2.5 μl $MgCl_2$ (2.5 m*M*), 0.1 μl of primer (100 p*M*), 2 μl of 100 μ*M* dNTPs and 1.5 unit of *Taq* polymerase. PCR amplifications were performed on a GeneAmp PCR system 2400 (PE Applied Biosystems) under the following conditions: a hot start at 94 °C for 1 min, followed by 27 cycles of three steps: denaturation at 94 °C for 1 min, annealing at oligo-specific temperature for 1 min, extension at 72 °C for 4 min, and a final extension for 7 min at 72 °C. The optimised oligo-specific annealing temperatures were, respectively, 42 °C $(GATA)_4$, 52 °C $(CAA)_5$, 54 °C $(GACA)_4$ and 62 °C $(CAG)_5$. The ISSR amplification products were mixed with loading buffer (0.005% each of xylene cyanol and bromophenol blue, as tracking dyes), run for 4 h on 2% agarose gels at a constant voltage (100 V) and detected using ethidium bromide. The molecular-weight size ladder Smartladder (Eurogentec) was used for band sizing.

Results and discussion

The study reported here was aimed at developing an efficient *in vitro* regeneration protocol for cauliflower plants to enable micropropagation and subsequent genetic and molecular studies.

After 3 weeks the hypocotyls cultured on induction medium containing 1 $mg \cdot l^{-1}$ of 2,4-D and 1 $mg \cdot l^{-1}$ Kin (SEIM) began to develop calli on the cut edges (655/680; Table 1). One should note that with different hormonal balances, usually with a lower 2,4-D concentration, many explants showed more than one type of formation, i.e. calli, caulogenesis or rhizogenesis. Roots and shoots were observed in 71.8% and 19.8% of explants, respectively, when 2,4 D concentration was reduced to 0.5 $mg \cdot l^{-1}$, and in 2% and 68% at 0.1 $mg \cdot l^{-1}$ of 2,4-D. Two types of calli appeared on each medium: the former was white and compact, whereas the latter was bright green and friable. After 3 weeks of culture, these calli were subcultured in different maturation media.

Neither the friable calli issued from media other than SEIM nor the compact ones produced somatic embryos when subcultured on any of the tested media (data not shown). The bright-green calli initiated on SEI medium became embryogenic at the next subcul-

Table 1 Qualitative and quantitative effects of 2,4-D (2,4-dichlorophenoxyacetic acid) and Kin (kinetin) concentrations on induction of cauliflower hypocotyl explants after 3 weeks of culture

2,4 D (mg/l)	Kin (mg/l)	Number of explants	Explants[a] exhibiting		
			Calli (%)	Caulogenesis (%)	Rhizogenesis (%)
1	1	680	96.3	0.8	0.9
1	0.5	656	71.6	19.8	71.8
1	0.1	719	68	68	2

[a] Explants may present several types of structure

Table 2 Texture of calli and embryogenic potential using different maturation media

2,4-D	Kin	Number of calli	Friable calli (%)	Embryogenic calli (%)
1	1	179	52	27.4
1	0.1	185	84.9	0
0.1	1	163	27	0

ture on SEMM (SEIM with a reduced concentration of sucrose 2% w/v; Table 2). On SEMM, more than 50% of calli were friable (93/179) and 27.4% of them became embryogenic (49/179).

To help distinguish them from zygotic embryos, some authors have called those embryos which arise asexually embryoids. Unfortunately, this term also denotes any structure that emerges in tissue cultures and resembles a proembryo. Though using the term embryoid, Pareek and Chandra had no evidence of such a development into a cauliflower embryo (1978). In the study reported here, embryo production started within 4 weeks of subculture of calli and continued even after 8 months (Fig. 1A). Thus, the experiments presented above showed, for the first time, that *Brassica oleracea* var. *botrytis* L. was able to regenerate via embryogenesis. Whereas Zhao et al. (1996) succeeded in *B. napus* embryogenesis from protoplasts or microspores, we performed it from explants through the formation of an embryogenic cell-line. Stable and regenerative embryogenic masses are a sought-after tool for obtaining clonal plants. However, the use of somatic embryos in commercial production depends on the implementation of an effective technique for obtaining high-quality somatic embryos at low cost. Indeed, the critical step in somatic embryogenesis is the maturation process, that is the ability to form embryos that will develop further to complete plantlets. In addition, cotyledon abnormalities can also occur: the present study showed that, out of 357 observed embryos, 42 produced multiple cotyledons, another 12 resulted in fused cotyledons and 9 gave unequally sized pairs. Even though the importance of ABA for differentiation following germination of somatic embryos is known in the *Brassica* genus, the embryos obtained in our study had germinated on growth regulator-free medium. Embryo development on medium without growth regulators has also been demonstrated for *B. campestris* (Bhattacharya and Sen 1980) and *B. juncea*. In our case, plants acclimatised well to the greenhouse conditions and were uniform; when they were transplanted to potting soil, they grew to maturity and produced seeds (Fig. 1C).

However, the quality of somatic embryogenesis rests on the production of true-to-type plants. Since molecular tools appear more reliable than phenotypic observations for evaluating variations, plant genome instability has been investigated by random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP; Brown 1989). Dallas (1988) and Nelke et al. (1993) assessed the genomic variations arising during *in vitro* cultures of rice and red clover by using minisatellite probes. ISSR markers are based on the specific amplification of genomic sequences by microsatellite primers. Applications have been reported in cultivar identification (Zietkiewicz et al. 1994; Wolff et al. 1995; Charters et al. 1996; Tsumara et al. 1996; Fang and Roose 1997; Nagaoka and Ogihara 1997; Blair et al. 1999), evaluation of genetic diversity (Kantety et al. 1995; Parsons et al. 1997; Struss and Plieske 1998), mapping (Kojima et al. 1998) and characterisation of agronomic markers (Akagi et al. 1996; Gold et al. 1999).

Characterisation of somaclonal variation has opened up a new field of application for this technique (Leroy et al. 2000). In this previous work, we demonstrated the usefulness of such markers for detecting genomic events. If we analyse our ISSR data to estimate the number of mutations by insertions or deletions, we can evaluate their number as ([number of size-altered bands/total size of the PCR fragments] × genome size). At least 5×10^4 individual genomic events have occurred in one callus.

Here, four microsatellite-primers were used for fingerprinting each plant or callus. The stability of patterns was investigated with two trinucleotides $(CAG)_5$ and $(CAA)_5$, and two tetranucleotides $(GACA)_4$ and $(GATA)_4$; these primers were chosen because of their previous use in the determination of genome instability through a callus phase (Leroy et al. 1997). Here, all the 65 markers produced (Fig. 2) were monomorphic across the calli and plants regenerated after somatic embryogenesis (Table 3). Thus, these results confirm that the plants regenerated via somatic embryogenesis are usually less variable than those regenerated via shoot and root morphogenesis. The plants regenerated from somatic embryos contain few mutations or chimeras due to stringent internal genetic controls throughout embryo formation, causing selection pressure against abnormal types. Bao et al. (1996) observed culture-induced DNA changes in transgenic rice plants produced via protoplast transformation. Wang et al. (1996) applied microsatellite, RFLP and RAPD markers for genomic polymorphism analysis in transgenic poplar clones. They concluded that all poplar clones regenerated from *in vitro* culture showed genomic modifications by one or more of the three analytical approaches. But with an RAPD approach, Goto et al. (1998) found no difference in 36 shoots regenerated from a single plant. However, these techniques do not target fast-evolving sequences, suffer from reproducibility problems or are laborious and time-consuming. Microsatellite primers are proposed as an alternative to overcome the limitations of other techniques.

This research work demonstrated that somatic embryogenesis could take place in cauliflower. Visual analysis of plants did not reveal any morphological

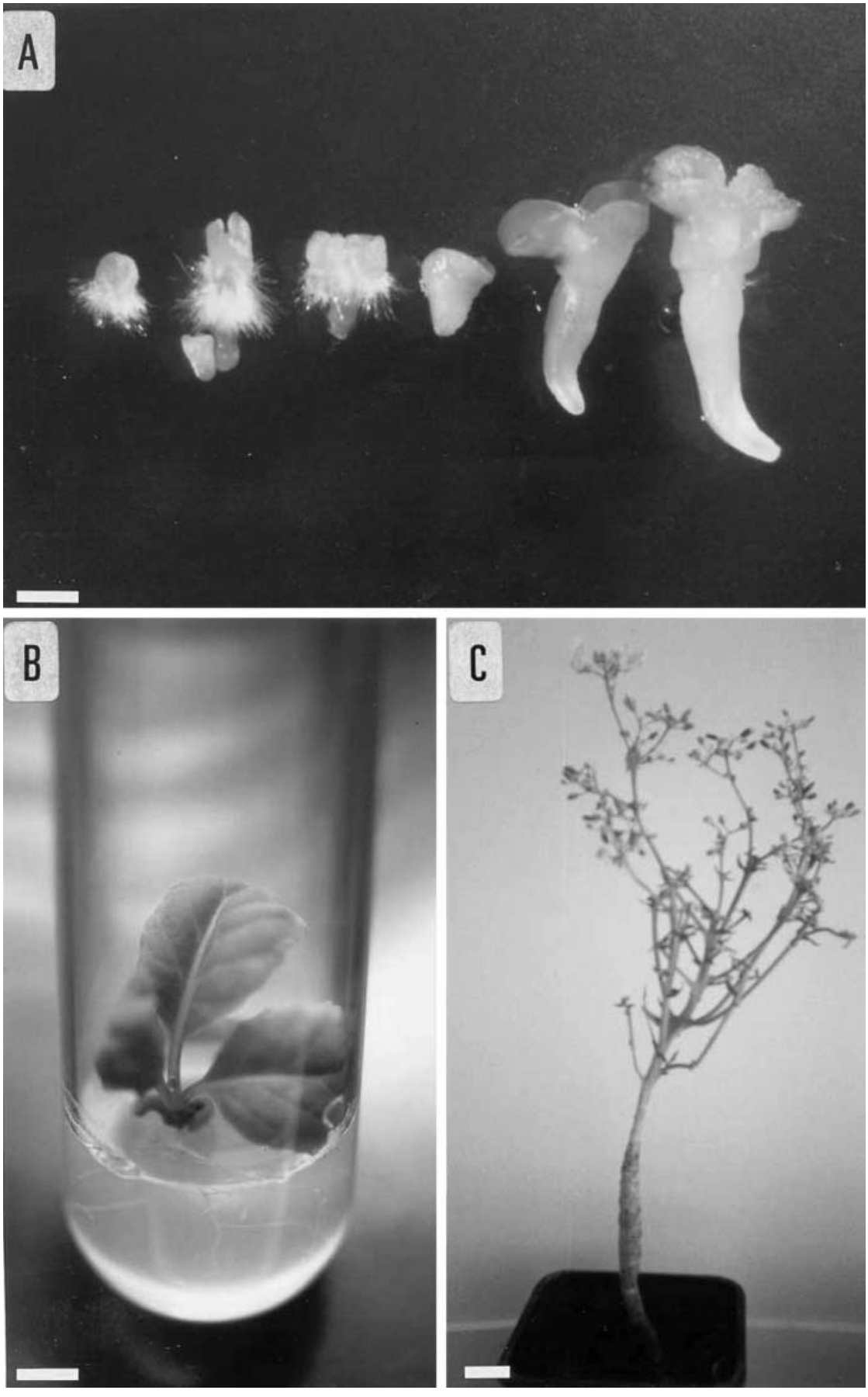

Fig. 1A–C Somatic embryogenesis and plant regeneration in a cauliflower tissue culture. **A** Different stages of isolated somatic embryos. Globular, early heart, late heart, torpedo stage embryos (*left to right*). *Scale bar* 3 mm. **B** Flowering plant transplanted to potting soil. *Scale bar* 1 cm. **C** Inflorescence derived from somatic embryogenesis plant. *Scale bar* 1.5 cm

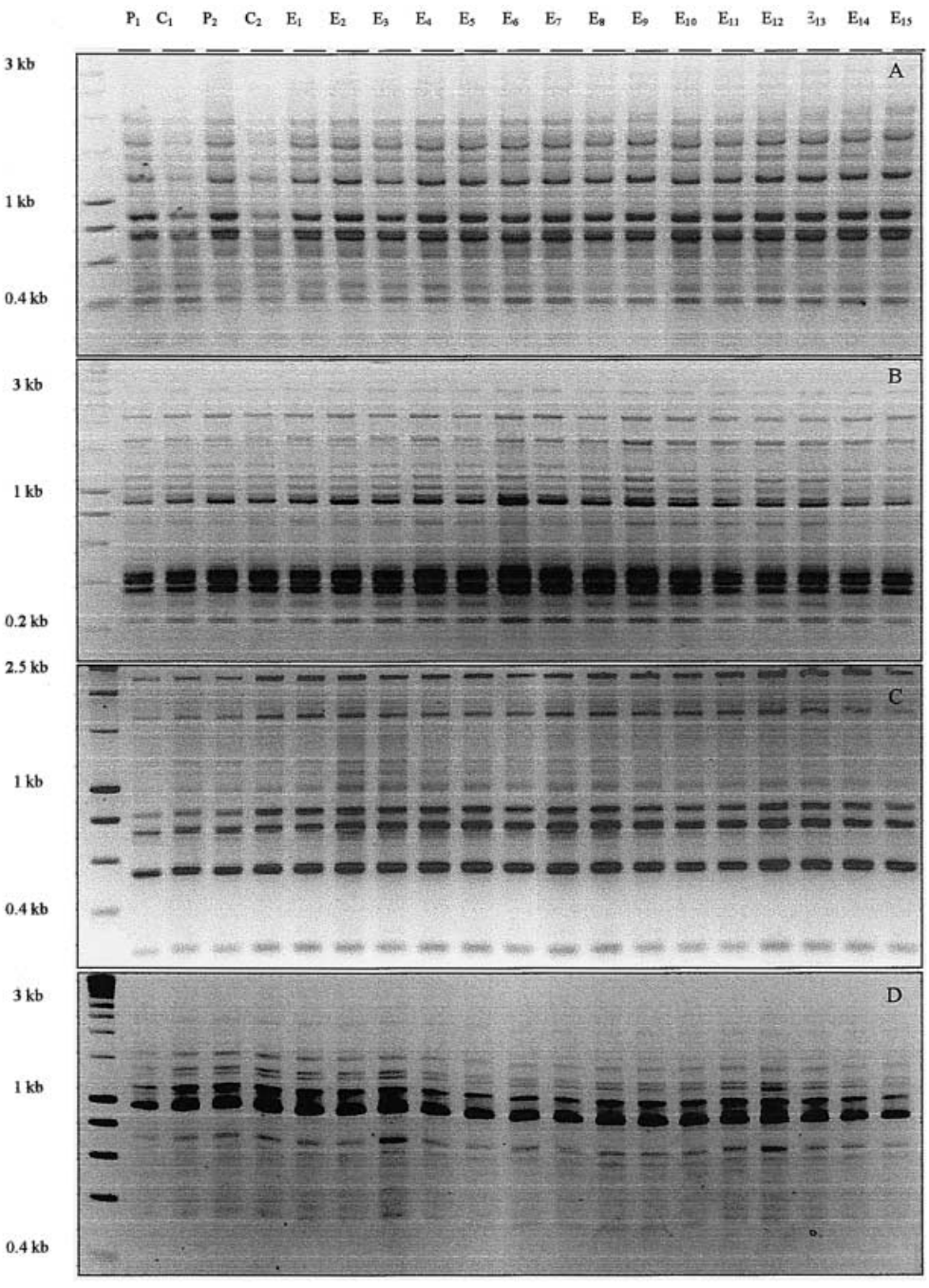

Fig. 2A–D PCR analysis of a subset of 15 cauliflower regenerants, mother plants and intermediate calli using SSR-primer. **A** $(CAA)_5$, **B** $(CAG)_5$, **C** $(GACA)_4$ and **D** $(GATA)_4$. Products were assayed on 2% agarose gel and stained with ethidium bromide. Molecular weight marker is Smart Ladder (Eurogentec); reference sizes are indicated in bp

Table 3 ISSR primer sequences used for cauliflower analysis with GC contents, annealing temperatures, number and size range of bands produced

Primer type	Primer sequence (5′–3′)	GC content (%)	Annealing temp. (°C)	bands amplified	MW size range (bp)
Tri-nt	CAACAACAACAACAA	33.3	52	18	400–2800
	CAGCAGCAGCAGCAG	66.6	62	17	250–3200
Tetra-nt	GATAGATAGATAGATA	25	42	12	200–3000
	GACAGACAGACAGACA	50	54	18	150–3500

aberrations. Investigation of polymorphism with ISSR markers confirmed the stability of the genetic material through the clonal phase. However, the comparison of our results with those obtained on cauliflower calli (data not shown) suggests that studies should be deepened to get a better idea of the relationships between plant regeneration protocols and mutation frequency.

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