Characterization of a defensin gene expressed in oil palm inflorescences: induction during tissue culture and possible association with epigenetic somaclonal variation events

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Abstract

From differential display studies performed on oil palm (Elaeis guineensis Jacq.) tissue cultures bearing or lacking an epigenetic homeotic flowering abnormality, known as mantled, EGAD1, a gene coding for a putative plant defensin, has been identified and characterized. In whole plants, transcripts of the EGAD1 gene were detected only in inflorescences. The closest characterized relative of the oil palm EGAD1 gene is the Petunia PPT gene, which is expressed principally in the pistil of the flower. The 77 amino acid polypeptide encoded by the EGAD1 gene displays strong similarities with a number of plant defensin proteins, which are thought to play a protective role and which have been shown in some cases to possess antifungal properties. Oil palm tissue cultures exhibit a generally strong induction of accumulation of EGAD1 transcripts, which were detected to differing extents at all stages of the tissue culture regeneration process. The 5' flanking region of the EGAD1 gene was found to contain two different types of potential cis-acting DNA element previously identified in the promoters of plant defence-related genes, which may explain the observed expression in tissue cultures. At the callus stage of the in vitro regeneration procedure, a differential accumulation of EGAD1 transcripts was observed which correlated with the presence or absence of the mantled flowering abnormality. EGAD1 gene expression may therefore be a marker of epigenetic somaclonal variation events.

Key words: Defensin, Elaeis guineensis, flower abnormality, somaclonal variation.

Introduction

Higher plants have developed a range of systems by which to protect themselves from damage associated with biotic and abiotic stress. Certain developmental stages are particularly vulnerable and must be afforded extra protection in order to allow successful completion of the reproductive cycle. A good example is the flower, which often contains tissues rich in macromolecules which may therefore be a target for invading herbivores or microrganisms (Lotan et al., 1989). In order to protect floral organs from attack by microbes, pests and herbivores, a range of protective molecules may be synthesized to provide a chemical defence, including chitinases (Leung, 1992), β-glucanases (Lotan et al., 1989), hydroxyproline-rich proteins (Chen et al., 1992), and proteinase inhibitors (Atkinson et al., 1993). The accumulation of defence-related proteins may be subject to both tissue-specific and environmental control, i.e. they may be activated as part of a predetermined developmental programme or be inducible. Considerable progress has been made in the last few years in the understanding of signalling pathways governing the activation of higher plant genes by biotic and abiotic stress (Maleck and Dietrich, 1999; Rojo et al., 1999; Kvtun et al., 2000; Meskiene et al., 1998; Liu et al., 1998).

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Although the defence systems of higher plants have evolved as an adaption to the demands of their natural environment, it is interesting to note that they may also be activated by conditions prevailing in tissue culture. This is particularly marked in protoplast culture, where the induction of a number of genes normally induced by biotic and abiotic stress has been observed (Yu et al., 1991). Although in the case of protoplasts, fungal extracts used to digest the cell wall may play a role in eliciting the observed response (Fleck et al., 1982), the activation of defence-related genes has also been observed in callus and cell cultures (Meyer, 1993). The inherent stresses associated with tissue culture are likely to play an important role in the induction of somaclonal variation (Larkin and Scowcroft, 1981; Karp, 1991) and it is particularly interesting to note that the activation of plant retrotransposons by tissue culture is mediated by promoter elements closely resembling those of plant defence-related genes (Grandbastien, 1998; Mhiri et al., 1997).

An interest in a flowering abnormality known as mantled (Corley et al., 1986), which is induced by tissue culture in oil palm (Elaeis guineensis Jacq.), has led to the study of gene expression patterns associated with somaclonal variation in this species. In vitro micropropagation based on somatic embryogenesis has been used to carry out the multiplication of elite oil palm genotypes for a number of years (Rival et al., 1996); however, the large-scale use of this approach is hampered by the occurrence of the mantled phenotype, which is observed on approximately 5–10% of all clonal palms regenerated in this way. The mantled phenotype involves a feminization of both male and female flowers, which in oil palm are produced alternately on the same plant. In abnormal male flowers, stamens develop as carpelloid structures, whilst in abnormal female flowers, the staminodes (vestigial stamens) develop as pseudocarpel structures. Although the defence systems of higher plants have evolved as an adaption to the demands of their natural environment, it is interesting to note that they may also be activated by conditions prevailing in tissue culture. This is particularly marked in protoplast culture, where the induction of a number of genes normally induced by biotic and abiotic stress has been observed (Yu et al., 1991). Although in the case of protoplasts, fungal extracts used to digest the cell wall may play a role in eliciting the observed response (Fleck et al., 1982), the activation of defence-related genes has also been observed in callus and cell cultures (Meyer, 1993). The inherent stresses associated with tissue culture are likely to play an important role in the induction of somaclonal variation (Larkin and Scowcroft, 1981; Karp, 1991) and it is particularly interesting to note that the activation of plant retrotransposons by tissue culture is mediated by promoter elements closely resembling those of plant defence-related genes (Grandbastien, 1998; Mhiri et al., 1997).

Materials and methods

Plant material

Oil palm tissue cultures were established and maintained as previously described (Pannetier et al., 1981). Two different clonal lines, LAB146 and LAB147 were used for differential display analysis. The LAB146 culture (normal; hereafter referred to as ‘N’) was obtained by direct cloning of a seed-derived palm and may therefore be assumed to carry little or no mantled abnormality (i.e. it should generate either a low percentage of mantled regenerants or only normal clonal progeny). The LAB147 culture (abnormal; hereafter referred to as ‘A’) was obtained by recloning a mantled tissue culture-derived palm and can therefore be assumed to produce 100% abnormal regenerants (Rival et al., 1996). For Northern analysis, a triplet of cultures kindly provided by MPOB (Malaysia), namely X1, X2 and X3, plus two further cultures produced in the Montpellier lab (N’ and A’) were used. Tissues were harvested corresponding to three developmental stages in the regeneration process: nodular callus, somatic embryos and 1 cm shoot apex-containing segments excised from leafy shoots. Inflorescence material of seed-derived and regenerant oil palms was kindly provided by La Me Station (Côte d’Ivoire) and MPOB. Root and leaf material was harvested from seed-derived greenhouse plants grown in Montpellier. For DNA extraction, leaf material from adult palms of several different genotypes (see below) was used.

Extraction and analysis of RNA and DNA

All standard cloning procedures used in this study were carried out essentially as described previously (Sambrook et al., 1989) unless otherwise indicated. Total RNA was extracted as described previously (Corre et al., 1996). RNA gel electrophoresis and Northern transfer were carried out using a Northern Max–Gly kit (Ambion Corporation). Membranes were hybridized with 32P-radiolabelled DNA probes obtained using the random priming method (Feinberg and Vogelstein, 1983). Genomic DNA was extracted as previously described (Rival et al., 1998) and analysed by Southern blotting and hybridization using standard techniques.
Differential display analysis

Differential display analysis was carried out using the primers and PCR conditions described earlier (Malhotra et al., 1998) with \([\alpha-32P]dATP\) as the radiolabel. After denaturing polyacrylamide gel electrophoresis, differential bands were excised and reamplified by conventional PCR using the same primers as were used for the differential display amplifications. Reamplified DNAs were blunt end cloned into the EcoRI site of the pBluescript SK-phagemid (Stratagene). Several clones were sequenced for each marker reamplification reaction and when more than one cDNA sequence was found to be cloned for a given marker, each cDNA was tested separately.

Construction and screening of cDNA library

A cDNA library of estimated average insert size 1.1 kb was constructed in the vector Lambda ZAPII (Stratagene) from 1 cm shoot apex-containing segments excised from leafy shoots of the LAB147 culture grown for 3 weeks on medium containing 10⁻³ M benzylaminopurine (BAP). Screening was carried out by plaque hybridization at 60 °C in a buffer containing 5× Denhardt’s solution, 6× SSC, 4 μg ml⁻¹ sheared salmon sperm DNA, and 0.5% SDS.

Isolation of EGAD1 gene 5’ flanking region

A PCR-based approach was used to amplify the genomic 5’ flanking region of the EGAD1 gene. Nested antisense oligonucleotide primers of respective sequence 5’-AAGAGCAGGAGATAGCTGGAAGCATTCGC-3’ and 3’-CCGAGTGTCCATCGCAACACACTAGC-3’ hybridizing to the 5’ end of the EGAD1 cDNA were used for this purpose in conjunction with a GenomeWalker™ kit (Clontech). The resulting amplification product was cloned into the plasmid vector pCRII-TOPO (Invitrogen) and sequenced.

Results

Characterization of an EGAD1 cDNA fragment obtained by differential display

Differences in mRNA accumulation between leafy shoot segments of the N (normal) and A (abnormal) cultures were studied using differential display, enabling the identification of differential bands which were subsequently excised, reamplified by PCR and cloned. A total of 53 primer combinations were tested, allowing the identification of 46 differential bands, from which 58 different cDNA clones were obtained following PCR reamplification and cloning. The fact that cDNA fragments of more than one kind were obtained for some markers is presumed to be due to the presence of co-migrating DNA species in the differential display gel (Ziegzouti et al., 1997). One of the abnormal-specific cDNA fragments obtained, provisionally named m5B, was shown by Northern hybridization to correspond to an mRNA of c. 0.6 kb which accumulated at higher levels in abnormal leafy shoot segments compared with those obtained from the normal culture (Fig. 1). In a preliminary characterization of the tissue specificity of the m5B mRNA, it was found that the difference in accumulation of the 0.6 kb transcript between normal and abnormal cultures was even more marked at the callus stage than in the leafy shoot segments. Little or no difference in signal intensity was observed at the somatic embryo stage. In whole plants, the 0.6 kb transcript was found to be present in both normal and abnormal female inflorescences (25 cm and 30 cm, respectively, in length) harvested from clonal palms, similar signal levels being observed in each case. No m5B-specific signal was observed for leaves or roots of intact seedling plants. Overall, it was concluded that the gene represented by the m5B cDNA is expressed principally in inflorescence tissues in the intact plant, but that the m5B mRNA is also accumulated in tissue cultures in a potentially mantled-dependent/stage-specific fashion.

Isolation and characterization of the full length EGAD1 cDNA

The cDNA insert in the m5B clone was used as a probe to screen an oil palm cDNA library prepared from leafy shoot segments as described above. This allowed the purification and sequencing of six positive clones containing the previously determined m5B nucleotide sequence. A database search revealed that each of the cDNAs thus obtained codes for a cysteine-rich polypeptide sharing close similarities with the plant defensin (or γ-thionin) proteins (Terras et al., 1995). Although slight variations in size were observed between the cloned cDNAs, all appeared to correspond to the same gene. The longest cloned cDNA obtained, m5B-7, was selected for use in subsequent experiments and renamed EGAD1. The nucleotide sequence of the 535 bp EGAD1 cDNA (deposited in the Genbank database under the accession
number AF322914) and deduced sequence of the polypeptide for which it codes are shown in Fig. 2. EGAD1 codes for a 77 amino acid polypeptide of predicted size 5.3 kDa. The EGAD1 cDNA sequence shown in Fig. 2 is assumed to contain a full length coding region, since it includes a putative translation initiation codon which is conserved amongst plant defensins and which is preceded in the same reading frame by a TAG stop codon 12 bp further upstream. The polypeptide encoded by the EGAD1 gene contains a putative signal peptide sequence of 30 amino acids which is likely to be responsible for targeting the nascent polypeptide into the secretory pathway. In order to examine amino acid sequence conservation between the EGAD1 precursor and those of other defensin/γ-thionin proteins, a CLUSTAL alignment was carried out (Fig. 3). The EGAD1 precursor sequence was aligned, using the CLUSTAL W program (Thompson et al., 1994) with those of its three closest published relatives, namely the products of the genes PPT from Petunia inflata (Karunananda et al., 1994), J1-1 from Capsicum annuum (Meyer et al., 1996) and P322 from potato (Stiekema et al., 1988). The closest identified relative of EGAD1 is PPT, the two respective sequences sharing 63.6% identical residues. It is interesting to note that the positions of the eight cysteine residues are absolutely conserved between all four polypeptides, strongly suggesting that they all share the same secondary structure.

Estimation of the number of EGAD1-like gene loci present in the oil palm genome

Oil palm genomic DNA was extracted from leaves of three different adult oil palms, X, Y and E, of different genotype in each case (Note: palms X and E were used to obtain the tissue cultures X1 and N, respectively). DNAs were digested with three different restriction enzymes, Southern blotted and hybridized to the EGAD1 cDNA. The resulting autoradiograph (Fig. 4) suggests that the EGAD1 gene occurs as a single copy per haploid genome. All three restriction enzymes used (none of which cuts the EGAD1 cDNA) were found to produce only one hybridizing fragment, except in the case of EcoRI when used with DNA of the Y genotype; in this case, a doublet was observed. One possible explanation for the latter result is that it might be caused by a difference in restriction pattern between allelic regions in or bordering the EGAD1 gene locus in the Y genome.

Developmental expression of the EGAD1 gene in male and female oil palm inflorescences

Given that the EGAD1 gene appears to display an essentially inflorescence-specific expression pattern in the intact plant, a more detailed characterization of transcript accumulation was carried out during floral development. A range of different developmental stages, classified according to inflorescence length, was investigated for both male and female inflorescences harvested

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Fig. 2. Nucleotide sequence of the EGAD1 (m5B-7) cDNA and deduced sequence of the encoded polypeptide. The position of the putative signal peptide sequence is shown by underlining. The position of the putative translation termination codon is indicated by an asterisk.
from seed-derived palms. Additionally, 9 cm normal and abnormal male inflorescences of regenerant palms were included in the experiment in order to assess whether \( \text{EGAD1} \) transcript accumulation might be mantled-dependent in male floral tissue. In each case, total RNA was extracted from whole spikelets. Northern blotting and hybridization was carried out as for the tissue culture material. The results obtained are shown in Fig. 5. It can be seen that the accumulation of \( \text{EGAD1} \) transcripts is essentially constant for most of the duration of female inflorescence development, although it finally peters out at the 35 cm stage. In male inflorescences, transcript accumulation shows a more marked temporal regulation, with strongest expression occurring at the earliest stages of development. Transcript abundance remains essentially constant up to the 8 cm stage, after which it decreases, little or no signal being visible at the 20 cm stage. Comparison of \( \text{EGAD1} \) transcript accumulation in normal and abnormal male inflorescences of regenerant palms revealed that, as with their female counterparts, expression is apparently mantled-independent.

**Investigation of mantled-dependent expression of \( \text{EGAD1} \) in other oil palm genotypes and cultures**

Since the Northern hybridization data shown in Fig. 1 revealed a strong differential accumulation of \( \text{EGAD1} \) transcripts at the callus stage of tissue culture regeneration, this phenomenon was investigated further. The \( N \) and \( A \) cultures used for the differential display and preliminary expression analysis were not of identical genotype; thus it was important to check that the difference in transcript levels seen was not simply attributable to differences in genetic background. Therefore, \( \text{EGAD1} \) transcript accumulation was investigated in genotypically identical cultures differing only in their mantled status, namely the cultures \( X1 \) (normal), \( X2 \) (intermediate) and \( X3 \) (abnormal). These cultures were initiated from, respectively, a normal seed-derived palm, a normal regenerant palm previously cloned from the seed-derived palm and an abnormal regenerant palm again previously cloned from the same original palm. The \( X1 \), \( X2 \) and \( X3 \) cultures were all initiated at the same time, so as to eliminate any possible effects due to culture age. In terms of abnormality, the \( X2 \) culture can be considered as representing an intermediate situation in that it has been initiated from a normal palm, but consists of cells which have undergone an extra round of tissue culture compared with the \( X1 \) culture. In the same experiment, in order to check the reproducibility of \( \text{EGAD1} \) expression between different cultures obtained from a given starting material, callus lines \( N' \) and \( A' \) were analysed, which were respectively initiated from the
same palms as the N and A cultures. Figure 6 shows the Northern hybridization profiles obtained with each callus line using the EGAD1 probe. The hybridization profiles of the N' and A' samples are consistent with those shown in Fig. 1 for the N and A lines, thus confirming the reproducibility of these results between cloning experiments and providing further evidence that the differential EGAD1 gene expression patterns observed may be associated with the mantled abnormality. This hypothesis is further strengthened by the hybridization profile observed for the X1, X2 and X3 cultures, which differ in their mantled status within a common genotypic background. As expected, the X3 (abnormal) culture produces a dramatically stronger signal than the X1 (normal) culture. It is moreover interesting to note that the X2 (intermediate) culture produces a signal greater than that of X1, but weaker than that of X3, presumably reflecting the extra cycle of tissue culture ‘history’ which it carries compared with the X1 line.

Characterization of the EGAD1 genomic 5’ flanking region and identification of potential cis-acting promoter elements

In order to identify genomic DNA sequence motifs which might be involved in determining the observed inflorescence-specific and tissue culture-induced profile of transcript accumulation for EGAD1, a DNA fragment containing 1 kb of 5’ flanking sequence (deposited in the Genbank database under the accession number AF451325) was isolated by inverse PCR. The nucleotide sequence of the 600 bp genomic region preceding the position of the 5’ terminus of the EGAD1 cDNA clone is shown in Fig. 7. A search of the PLACE database (http://www.dna.afrc.go.jp/htdocs/PLACE) revealed the presence in the EGAD1 5’ flanking region of two different types of sequence motif potentially involved in stress- or defence-related regulation. Firstly, a W box-like TTTGACT element was identified at −511 (with respect to the 5’ end of the cDNA sequence). The W box is a cis-acting element found in the promoters of the parsley defence-related Pathogenesis-related Class10 (PR-10) and ELI17 genes (Eulgem et al., 1999; Kirsch et al., 2001). Secondly, two different CCGTCC elements resembling the A box found in several parsley PAL (phenylalanine ammonia lyase) gene promoters (Logemann et al., 1995) were identified at −396 and −226, respectively, in the EGAD1 gene 5’ flanking sequence. In addition to these two types of motif resembling stress- or defence-related cis-acting elements, a TATATAA sequence which might act as a TATA box transcription initiator was identified 52 bp upstream from the cDNA 5’ terminus. No conserved DNA motifs characteristic of the promoters of florally expressed genes were identified.

Discussion

The deduced polypeptide encoded by the EGAD1 gene product displays similarities with a range of different defensin type proteins identified in both monocotyledonous and dicotyledonous plants. Defensins were originally known as α-thionins as they share structural similarities with the α- and β-thionins (Garcia-Olmedo et al., 1989); in particular, they are of similar size (5 kDa)
Gene expressed mostly in floral tissues is of the plant. An additional example of a plant defensin proteins play with respect to the reproductive apparatus presumably reflects the protective role which the defensin cell layers of the floral organs. This expression pattern tissue-specific expression pattern within the flower, transcribed relative, the Petunia PPT gene, is expressed principally in the pistil of the flower (Karunanandaa et al., 1995). Defensins are thought to play a role in pathogen defence and in some cases have been shown to exert an antifungal action, brought about by electrostatic interaction with hyphal cell membranes (Thevissen et al., 1996).

Given that EGAD1 transcripts appear to accumulate mainly in inflorescence tissues in the intact plant, it is particularly interesting to note that its closest characterized relative, the Petunia PPT gene, is expressed principally in the pistil of the flower (Karunanandaa et al., 1994). The similar expression patterns of these two genes suggest an equivalent role for the PPT and EGAD1 proteins in protecting the reproductive organs of the plant from attack by microorganisms. This hypothesis is strengthened by studies performed on two other defensin genes expressed in flowers, FST from tobacco (Gu et al., 1992) and AT2 from tomato (Brandstädt et al., 1996). In both cases, in situ hybridization revealed a strongly tissue-specific expression pattern within the flower, transcript accumulation being localized mainly in the outer cell layers of the floral organs. This expression pattern presumably reflects the protective role which the defensin proteins play with respect to the reproductive apparatus of the plant. An additional example of a plant defensin gene expressed mostly in floral tissues is SD2 from sunflower (Urdangarin and de la Canal, 2000). It will be interesting to determine whether EGAD1 displays a tissue-specific localization of expression within the oil palm flower comparable to that observed for the other defensin genes mentioned. More generally, the various flrally-expressed defensin genes described above are likely to play similar biological roles and it will thus be interesting to compare their modes of regulation to determine whether they involve similar signalling pathways.

Previous studies have demonstrated that some plant defensin genes may be induced by wounding or pathogen attack. In Arabidopsis, the PDF1.2 gene encoding a defensin protein was observed to undergo a strong increase in expression in response to challenge by the fungal pathogen Alternaria brassicicola, the induction apparently occurring through a salicylic acid-independent pathway (Penninckx et al., 1996; Mitter et al., 1998). In immature fruits of Capsicum annuum, accumulation of the J1 defensin protein was found to be wound-inducible (Meyer et al., 1996); increases in the amounts of this protein also being found to occur during the ripening of the fruit. Defensins seem to be widespread in their tissue distribution, other members of this protein family accumulating in seeds (Colilla et al., 1990; Bloch and Richardson, 1991; Choi et al., 1995) and stem tubers (Stiekema et al., 1988). Although this study of EGAD1 expression has revealed that transcripts of this gene accumulate in a developmentally defined fashion in the inflorescence of whole plants, a further question to be addressed is whether a separate inducible mechanism might occur, explaining the detection of mRNA in oil palm tissues cultured in vitro. Tissue culture appears in this case to exert an antifungal action, brought about by electrostatic interaction with hyphal cell membranes (Thevissen et al., 1996).

Potential cis-acting regulatory elements in the genomic 5’ flanking region of the EGAD1 gene. The nucleotide sequence of the 600 bp region preceding that covered by the EGAD1 cDNA clone is shown. Numbering is respect to the position of the 5’ terminus of the cDNA clone. Three types of DNA motifs potentially involved in transcription regulation were identified: a ‘W box’-like TTTGACT element (Eulgem et al., 1998; Kirsch et al., 2001) present at –511 is indicated by the symbol ‘+’; two CCGTCC elements located at –396 and –226, respectively, which resemble the parsley PAL (phenylalanine ammonia lyase) gene promoter A box (Logemann et al., 1995) are marked by double underlining; and a TATATAA sequence at –52 which might act as a TATA box transcription initiator is labelled by single underlining.
search made it possible to identify two classes of DNA sequence motif which might be involved in stress- or defence-related regulation: a WRKY/ELI7 promoter W box-like element (Rushton et al., 1996; Eulgem et al., 1999; Kirsch et al., 2001) at −511 and two PAL promoter A box-like elements (Logemann et al., 1995) at −396 and −226. It is interesting to note that the parsley PAL, PR-10 and ELI7 genes are all induced by fungal elicitors.

The presence in the EGAD1 promoter of two different types of sequence motif characteristic of plant defence-related genes is not unexpected, given the biological role of defensin proteins and the observed induction of EGAD1 transcript accumulation during tissue culture. Although no potential inflorescence- or flower-specific DNA motifs were identified in the EGAD1 5′ flanking region, it is nevertheless possible that cis-acting elements of this type may be present in a form which is poorly conserved with respect to database sequences.

The fact that EGAD1 gene transcripts accumulate in significantly greater quantities in callus cultures initiated from mantled palms compared with those obtained from normally flowering individuals suggests a possible association between defensin gene expression and epigenetic somaclonal variation events at this stage in the in vitro regeneration process. This differential expression pattern might be explained either by a direct tissue culture-induced alteration of the activity of the EGAD1 promoter, or alternatively by the perturbation of the activity of regulatory genes acting further upstream in the same signalling pathway. A helpful approach in resolving this question will be the use of promoter::reporter gene constructs to carry out functional studies. Work of this type should also enable the resolution of promoter regions responsible for inflorescence-specific expression and tissue culture induction.

Recent studies suggest that the epigenetic alterations which determine the mantled flowering abnormality might include changes in genomic DNA methylation, a well-characterized phenomenon associated with somaclonal variation (Kaeppler and Phillips, 1993). The hypothesis of an involvement of DNA methylation is particularly attractive, since it is compatible with the observed spontaneous reversion to normal flowering. Indeed, recent work has demonstrated that the occurrence of the oil palm mantled phenotype is correlated with both global and sequence-specific genomic DNA hypomethylation (Jaligot et al., 2000; Matthes et al., 2001). So far, however, it has not been possible to identify a common genomic target sequence which is altered in all mantled palms. Given the highly specific nature of the abnormal flowering phenotype, it seems likely that only a small number of specific genes is affected. One possible explanation for the common occurrence of mantled palms in clonal progenies is the existence of specific DNA sequences in the oil palm genome which show ultra-sensitivity to tissue-culture induced methylation changes. Further studies of the molecular mechanisms underlying epigenetic modifications induced by tissue culture should improve current understanding of this phenomenon and provide tools for monitoring and controlling somaclonal variation.

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