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## Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants

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**Abstract** The propagation of plants through tissue culture can induce a variety of genetic and epigenetic changes. Variation in DNA methylation has been proposed as a mechanism that may explain at least a part of these changes. In the present study, the methylation of tomato callus DNA was compared with that of leaf DNA, from control or regenerated plants, at *MspI/HpaII* sites around five middle-repetitive sequences. Although the methylation of the internal cytosine in the recognition sequence CCGG varied from zero to nearly full methylation, depending on the probe used, no differences were found between callus and leaf DNA. For the external cytosine, small differences were revealed between leaf and callus DNA with two probes, but no polymorphisms were detected among DNA samples of calli or DNA samples of leaves of regenerated plants. When callus DNA cut with *HindIII* was studied with one of the probes, H9D9, most of the signal was found in high-molecular-weight DNA, as opposed to control leaf DNA where almost all the signal was in a fragment of 530 bp. Also, an extra fragment of 630 bp was found in the callus DNA that was not present in control leaf DNA. Among leaves of plants regenerated from tissue culture, the 630-bp fragment was found in 10 of 68 regenerated plants. This 630-bp fragment was present among progeny of only 4 of these 10 plants after selfing, i.e. it was partly inherited. In these cases, the fragment was not found in all progeny plants, indicating heterozygosity of the regenerated plants. The data are interpreted as indicating that a *HindIII* site becomes methylated in callus tissue, and that some of this methylation persists in regenerated plants and is partly transmitted to their progeny.

**Key words** Callus · Epigenetic variation · *Lycopersicon esculentum* · Plant regeneration · Somaclonal variation

### Introduction

Tissue-culture propagation can induce genetic and epigenetic changes in regenerated plants. Genetic changes have been coined 'somaclonal variation' by Larkin and Scowcroft (1981). This phenomenon has been reviewed extensively (e.g. Karp 1991), but insight into the process(es) leading to this variation is still limited. This is partly due to the variety of changes that can occur, ranging from point mutations to chromosome breakage and rearrangements (De Klerk 1990). In addition, it is also caused by the occurrence of changes that cannot be easily explained. These include mutations that are stable in a regenerated plant and during clonal propagation, but are not inherited by offspring; and changes that are inherited in selfed progeny, but disappear after crossing with a normal plant (Karp 1991).

Variation in DNA methylation has been proposed as a mechanism that may explain the wide range of changes that can occur after tissue-culture propagation, and might explain at least some of the peculiar changes described above (Kaeppeler and Phillips 1993; Smulders et al. 1995). Methylation of DNA bases can lead to repression of gene expression (Matzke and Matzke 1991), so a change in methylation may mimic a point mutation in its phenotypic effect, namely the loss of a functional protein. An increase in DNA methylation can also lead to a more condensed chromatin structure (Lewis and Bird 1991), which is replicated late in the cell cycle. This would increase the chances of chromosome breakage due to chromatid segregation during mitosis if DNA replication is not yet completed (Kaeppeler and Phillips 1993). In this way, changes in methylation may lead to deletions and inversions of parts of chromosomes.

The pattern of DNA methylation changes throughout the life cycle of a plant or animal (Brown 1989; Anderson et al. 1990). For instance, in tomato seeds 27.4% of the cytosine residues are methylated (mC). This drops to 20–21% in the young seedling. Mature leaves have 25.0% mC, but in pollen it is as low as 21.9% (Messeguier et al. 1991).

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From these data, it can be concluded that changes in overall methylation occur at least once in the life-cycle of a tomato plant. Also in wheat (Brown 1989; Brown et al. 1989) and pea (Watson et al. 1987) the methylation level drops when young seedlings develop. In mammals, it is known that the methylation pattern can be erased in the germ line (Holliday 1990). If this is also the case in plants, it may explain how a 'mutation' that consists of a change in methylation can be somatically stable but revert to wild-type in the progeny of a regenerated plant.

During the *in vitro* phase, the overall methylation percentage in maize is reduced (Kaepler and Phillips 1993). In tomato, protoplasts contained 20.1% mC, which was comparable to young seedling tissues and is among the lowest percentage found (Messegueur et al. 1991). Anderson et al. (1990) mostly found lower methylation of rRNA genes in petunia calli, but some acquired high levels of methylation. The 5s-rRNA genes in soybean callus also became less methylated although re-methylation can occur during the next 2 years (Quemada et al. 1987). In carrot root explants, DNA methylation initially decreased but was increased after 2 weeks in the presence of auxin (Lo-Schiavo et al. 1989; Arnholdt-Schmidt et al. 1991; Arnholdt-Schmidt 1993). The pea cultivar "Dolce Provenza" showed an increase in methylation after regeneration (Cecchini et al. 1992). In contrast, CmCGG methylation in repetitive DNA did not change during 9 months of *Cucumis melo* callus cultures (Grisvard et al. 1990) and in some repetitive DNA sequences in tobacco, while methylation of 25s-rDNA sequences decreased somewhat in callus and plants regenerated from tissue culture (Vyskot et al. 1993).

Overall changes in methylation imply changes in the methylation status of some restriction sites. Studying such sites, frequent changes were found in barley anther culture-derived double haploids (Duvaux et al. 1993). Changes in DNA methylation were also frequent in maize callus, regenerated plants, and the progeny of such plants (Brown et al. 1991), and these methylation changes were stably inherited for two generations (Kaepler and Phillips 1993). It was also found that some probes detected methylation changes more often than others, so perhaps methylation changes do not occur at random. Alternatively, some changes may be more stable than others. Maize is a monocotyledonous species in which structural changes are found at reasonable frequencies (De Klerk 1990; De Klerk and Bouman 1990; Karp 1991). In this species, the occurrence of transposon activity as a result of tissue culture (James and Stadler 1989; Peschke and Phillips 1991) is expected to contribute to this instability (Karp 1995).

The purpose of the present study was to analyze DNA methylation changes in tomato, a dicotyledonous species in which we were unable to detect any structural change in the DNA of calli and regenerated plants (Vosman et al. 1992; Rus-Kortekaas et al. 1994). The strategy employed was elaborated from the finding that leaves of regenerated plants do not show the DNA methylation pattern of the callus that they have been regenerated from, but have, in principle, switched back to the 'leaf' methylation pattern (Anderson et al. 1990). Because of this, we first selected

probe/enzyme combinations that showed a different methylation pattern in callus compared to the leaves of control plants. Then, the leaves of regenerated plants and their progeny were screened for the presence of any residual callus-type methylation.

## Material and methods

### Plant material

Control plants were grown in the greenhouse from seeds of *Lycopersicon esculentum* cv Moneymaker (CGN-collection). First-generation regenerated (R1) plants were grown in the greenhouse after rooting of regenerated shoots (see below). Some second-generation regenerated (R2) plants were grown from seeds obtained by selfing selected R1 plants. Other R2 plants were grown from seeds of R1 plants that had been regenerated following the same protocol (Van den Bulk et al. 1990). Young leaves were harvested from 2 to 3-month-old plants, and stored at  $-80^{\circ}\text{C}$  until DNA extraction. Each DNA extraction was done using material from one plant.

For comparison, the same pieces of leaf, cotyledon and hypocotyl tissues as used for tissue culture were collected from seedlings grown *in vitro*, and stored at  $-80^{\circ}\text{C}$  until DNA extraction. Material from approximately 50–100 seedlings was used for one DNA extraction.

### Tissue culture

Tissue culture was started from seedlings grown aseptically for up to 5 weeks on MS-medium (Duchefa) supplemented with 2% sucrose and 0.65% agar (BBL) in 720-ml glass jars in a growth room at  $24^{\circ}\text{C}$  and with  $16\text{ h day}^{-1}$  light (Philips fluorescent TL type 50 W 84HF, maximum distance 30 cm).

For the induction of regeneration, pieces of leaves, cotyledon and hypocotyl were cultured aseptically on MS-medium supplemented with 0.65% agar, 3% sucrose,  $0.6\text{ }\mu\text{M}$  IAA, and  $4.6\text{ }\mu\text{M}$  zeatin (Sigma) (Van den Bulk et al. 1990). After 6–9 weeks (leaf and cotyledon explants) or 9–12 weeks (hypocotyl explants), shoots were taken from the explants and rooted for 1–3 weeks on MS-medium containing 0.65% agar and 2% sucrose. After rooting, the regenerated plants were transferred to the greenhouse and grown to maturity.

To obtain callus, the leaf, cotyledon and hypocotyl explants were cultured on MS medium supplemented with 0.65% agar, 3% sucrose,  $11\text{ }\mu\text{M}$  of 1-naphthaleneacetic acid, and  $4\text{ }\mu\text{M}$  of benzylaminopurine. Callus was excised from cotyledon and hypocotyl explants after 6 weeks of culture and from leaf explants after 9 weeks. The callus material was subcultured at 3-week intervals for a period of 9 weeks. Individual calli were stored at  $-80^{\circ}\text{C}$  until DNA extraction.

### DNA extraction and digestion

DNA was extracted from calli and seedling organs according to Delaporta et al. (1983). The procedure was modified by adding 1 M NaCl to precipitate polysaccharides before the first DNA precipitation (Rus-Kortekaas et al. 1994). DNA of the R1 and R2 plants was extracted from young leaves according to the method of Bernatzky and Tanksley (1986) with modifications (Vosman et al. 1992).

For Southern hybridization, DNA was digested with the restriction endonucleases according to the manufacturer (Gibco/BRL), but with excess enzyme ( $10\text{ U}/\mu\text{g}$  of DNA). All digestions were repeated after a phenol-chloroform extraction. After the restrictions, the DNA was separated on a 0.8% or 1.7% agarose gel and alkaline-blotted overnight onto Hybond N<sup>+</sup> (Amersham).

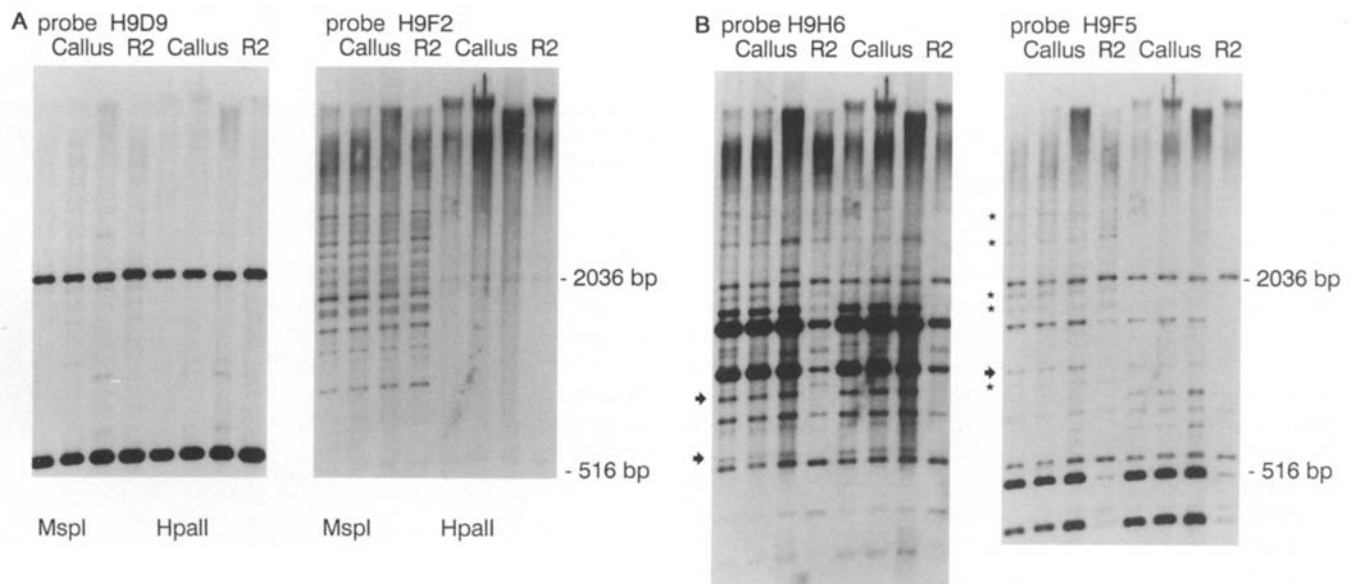
### Probes

The repetitive probes used in this study were isolated from a bank of *Hind*III-digested total DNA from *L. esculentum* 83M7138 in

**Table 1** Summary of polymorphisms detected between DNA of callus and the leaves of control Moneymaker plants by the repetitive probes used in this study

Probe	Size (bp)	Estimated copy number	Polymorphisms in restriction patterns			
			Between <i>Msp</i> I and <i>Hpa</i> II (CmCGG-methylation)		Between leaf and callus by both <i>Msp</i> I and <i>Hpa</i> II (mCCGG-methylation)	Between leaf and callus by <i>Hind</i> III
			in callus	in leaf		
H9D9	530	560	— <sup>a</sup>	—	—	+
H9E12	2900	3	+	+	—	—
H9F2	230	2580	+	+	—	—
H9F5	1000	26	+	+	+	—
H9H6	1600	15	—	—	+	+

<sup>a</sup> —, no polymorphism; +, polymorphism(s) present



**Fig. 1** *Msp*I digests (left four lanes) and *Hpa*II digests (right four lanes) of DNA from three individual calli and from young leaves of one regenerated plant (R2). The calli had been induced on cotyledon, hypocotyl and leaf material. The samples were separated on 0.8% agarose and blotted onto Hybond N+. This blot was hybridized with the probes H9D9, H9F2 (A), H9H6, and H9F5 (B). In B, stars mark some fragments that are specific for samples digested with *Msp*I, and arrows mark some fragments specific for callus DNA samples

pUC18 (a gift of P. Zabel, WAU, Wageningen, The Netherlands). The probes were selected for a medium-to-high copy number by hybridizing different dilutions of tomato genomic DNA to the inserts. As a reference, the same dilutions of genomic DNA were hybridized with the single-copy probe TG209 (insert size 950 bp). Some characteristics of the probes are listed in Table 1.

#### Hybridizations

The inserts were amplified by PCR using pUC primers, and random-primed labelled as recommended by the supplier of the DNA labelling kit (USB biochemicals). Hybridizations were performed overnight at 65°C in a hybridization buffer consisting of 1 M NaCl, 50 mM Tris-HCl pH 7.5, 1% SDS, 10% dextran sulphate, and 0.1 mg/ml salmon-sperm DNA (Sigma). Blots were washed at 65°C for 10 min in 2SSC, followed by two high stringency washes of 30 min in 0.1SSC, 0.1% SDS, at 65°C, and autoradiographed using KODAK

XAR-5 film at –80°C. For crucial comparisons among certain samples, the samples were processed together and placed on one blot.

## Results

### Methylation differences at *Msp*I/*Hpa*II sites

A number of repetitive probes were used to detect differences in DNA methylation between callus DNA and leaf DNA from control plants using *Msp*I- and *Hpa*II-digested DNA samples. With probe H9D9, no differences were found either between calli and leaf DNA or between the digestions with the two enzymes (Fig. 1). Apparently, there was no methylation of the internal cytosine at the CCGG sites made visible with this probe.

This was not so for all repetitive probes. When the same blot was hybridized with H9F2, the differences in banding patterns between the *Hpa*II and *Msp*I digestions indicated almost full methylation at the second C of the CCGG sites. Again, the patterns of callus and leaf DNA were identical

(Fig. 1). Using H9E12, the *HpaII* digestions were different from the *MspI* digestions, but only in a few bands, indicating some CmCGG methylation (data not shown). Differences between leaf and callus DNA in the methylation of the first cytosine (mCCGG) were found using probe H9H6, but no CmCGG methylation was detected (Fig. 1). Finally, probe H9F5 identified the presence of both types of methylation, and showed that mCCGG methylation differences were present between leaf and callus DNA (Fig. 1). These results are summarized in Table 1.

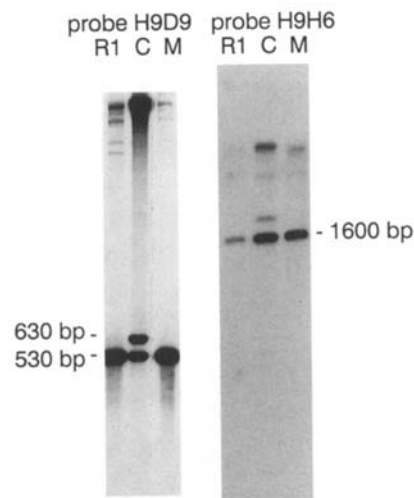
A number of these probes were subsequently used to see whether differences could be detected among leaf DNA samples from R1 and R2 plants digested with *MspI* or *HpaII*. No difference in banding pattern was observed among 57 R1 plants and 88 R2 plants (data not shown).

#### Differences between callus and leaf DNA in *HindIII* digests

As reported previously (Vosman et al. 1992; Rus-Kortekaas et al. 1994), RFLPs among DNA samples of plants regenerated from tissue culture have also been searched for in our laboratory. To this end, leaf DNA from a number of R1 or R2 plants was digested with the restriction enzymes *TaqI*, *EcoRI* or *HindIII* and hybridized to probes that visualized a number of bands per lane, including the oligonucleotides (GATA)<sub>4</sub> and (GACA)<sub>4</sub> (Vosman et al. 1992; Arens et al. 1995) and the repetitive DNA probe H9H6. With the latter probe, no difference was detected among 258 DNA samples of calli, and among 165 samples of R2 plant leaf DNA (data not shown).

Surprisingly, when callus and control or R1 leaf DNA samples digested with *HindIII* were hybridized with H9D9, clear differences were visible between the callus sample on the one hand and the leaf samples on the other hand. First, in the leaf samples almost all of the signal was in a fragment of the expected length of 530 bp, but in the callus samples most of the signal was found in the high-molecular-weight area. Second, the callus samples contained an extra fragment of approximately 630 bp that was not present in control leaf samples (Fig. 2). When the other repetitive probes were tested on *HindIII*-digested DNA from calli and leaves, all probes hybridized to the extra fragment in the calli samples, as well as to the 530-bp fragment in all samples, but at a much lower intensity. When the probes were first restricted with *HindIII* to remove the PUC poly-linker that was common to all probes, both bands disappeared from the hybridizations, except from those with probe H9D9 which were unaffected. Probe H9H6 visualized a band of approximately 1900 bp in the callus samples that was not present in leaf DNA (Fig. 2; results summarized in Table 1). In the next experiment, attention was focussed on the extra 630-bp fragment visualized by probe H9D9.

In total, DNA samples of leaf material of 174 plants (12 control Moneymaker plants, 68 R1 plants, and 94 R2 plants), as well as DNA samples of 69 calli, were *HindIII*-digested and hybridized with H9D9. The 630-bp fragment



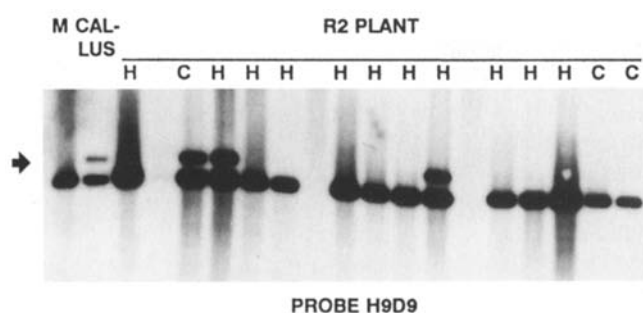
**Fig. 2** *HindIII* digests of DNA from leaf material of a seed-propagated tomato cv Moneymaker plant (M), one callus (C), and leaves of one regenerated plant (R1). The samples were separated on 0.8% agarose, and blotted onto Hybond N+. The blots were hybridized with H9D9 and H9H6

was present in all callus samples, and the callus hybridization pattern could thus always be recognized. All leaf material from control Moneymaker plants, as well as the leaf samples of most regenerated plants, lacked the 630-bp band. However, a few regenerated plants did contain the 630-bp fragment.

#### Differences in *HindIII* digests are not due to partial digestion

Our working hypothesis is that the extra 630 bp fragment is the result of DNA methylation at one of the *HindIII* restriction sites flanking the 530-bp fragment in the callus DNA. *HindIII* is inhibited by mC (5-methylcytosine) and mA (5-methyladenine), i.e. it does not cut AAGmCTT (Huang et al. 1982) or mAAGCTT (McClelland and Nelson 1985). Although no isoschizomers are known for *HindIII*, four lines of indirect evidence indicate that the 630-bp fragment is not the result of partial digestion of all the callus DNA samples and a few leaf samples.

- (1) The callus DNA extracted by our procedure could be restricted by several enzymes: *TaqI*, *EcoRI*, and *HinfI*. The hybridization patterns obtained with DNA restricted with any of these enzymes and the repetitive probes H9D9, H9E12, H9F2, H9F5 or H9H6, as well as oligonucleotide probes, were identical for callus and leaf samples (data not shown). Also *MspI*- and *HpaII*-restricted leaf and callus samples gave identical patterns with, for example, H9D9 (Fig. 1).
- (2) When the same *HindIII* blots were hybridized with H9E12, H9F2 or H9F5, no differences were visible among the leaf and callus samples.
- (3) When DNA was extracted from different organs of the same plant (root, hypocotyl, cotyledon, older and young



**Fig. 3** *Hind*III digests of DNA of leaf material of a seed-propagated tomato cv Moneymaker plant (M), an individual callus, and a number of R2 plants derived from cotyledon (C) or hypocotyl (H) explants. Blot hybridized with H9D9. The arrow indicates the extra 630-bp fragment that can be found in the callus samples and in some of the R2 plants

**Table 2** Presence of the extra 630-bp fragment in *Hind*III-restricted DNA from direct regenerants (R1) and the R2 progeny of selected R1 plants, hybridized with H9D9

Ploidy-level	2n			4n			Total
Origin	Leaf	Cotyl	Hypo-cotyl	Leaf	Cotyl	Hypo-cotyl	
R1 plants							
– <sup>a</sup>	24	26	0	2	5	1	58
+	1 <sup>b</sup>	0	0	0	0	9	10
Total	25	26	0	2	5	10	68
R2 progeny of ‘+’ R1 plants <sup>c</sup>							
–	1	0	0	0	0	23	24
+	2	0	0	0	0	4 <sup>d</sup>	6

<sup>a</sup> –, fragment absent; +, fragment present

<sup>b</sup> A different polymorphism: 450-bp fragment in stead of 630-bp fragment

<sup>c</sup> Three progeny plants were tested for each '+’ R1 plant

<sup>d</sup> From three different R1 parents

leaves of tomato seedlings) and restricted with *Hind*III, *Eco*R1, or *Msp*I, the hybridizations with H9D9 yielded one pattern for each enzyme, irrespective of the tissue used (data not shown). Conversely, some leaf samples of R1 and R2 regenerated plants (see below) did show the extra band. Therefore, the presence of an extra fragment does not correlate with a certain type of tissue.

(4) Treating callus DNA samples with proteinase K before *Hind*III restriction did not remove the 630-bp fragment from the hybridization pattern obtained with H9D9, indicating no interference by proteins (data not shown).

#### Variation detected among regenerated plants and their progeny

Among the 68 R1 plants that were studied with probe H9D9 using *Hind*III-digested DNA, ten plants (15%) showed an extra fragment (Table 2). In nine of the ten plants, the

630-bp fragment characteristic of callus samples was found (examples are shown in Fig. 3). These nine plants were all tetraploids regenerated from hypocotyl tissue. One diploid plant regenerated from leaf tissue also showed a polymorphism, but this sample contained a smaller fragment of approximately 450 bp (data not shown).

To study the inheritance of the changes, the R1 plants were allowed to self-pollinate and set seed. Three R2 seedlings of each of the ten R1 plants that had shown a polymorphism were grown in the greenhouse. After 2 months, when these seedling plants had developed at least two mature leaves, DNA was isolated from the young leaves, restricted with *Hind*III, and analyzed with probe H9D9. The extra fragment was found among the progeny of 3 of the 9 plants regenerated from hypocotyl tissue: in one case in 2 of the 3 R2 plants of the same parent, and in the other cases in only 1 of 3 R2 plants. For the plant regenerated from leaf tissue, two progeny plants showed a polymorphism. Surprisingly, this was the same 630 bp fragment as in the other plants. It is, therefore, not clear what has caused the 450-bp band in the regenerated plant.

Thus, four of the ten progeny lines were found to contain a polymorphism. The finding that progeny were recovered with and without the extra fragment is consistent with the fragment being present in the tissue culture-derived R1 plants in a heterozygous form. Theoretically, in the case of a tetraploid plant with only one of the four chromatids changed, random testing of three progeny plants reduces the chance of not detecting the polymorphism to 3.1%. From this it was calculated that, among the six negative progenies, one positive progeny line may have remained unnoticed ( $P > 0.05$ ). Thus, the inheritance of the fragment to the second generation can be estimated to be 40–50%. The ten control Moneymaker plants, and the majority of the R1 and R2 plants, did not show the polymorphism. If we assume that the polymorphism is indeed the result of tissue-culture propagation, and will not appear in progeny of the 59 plants that did not show the polymorphism themselves, then the overall frequency of this polymorphism in the second-generation plants regenerated from tissue culture is 6–7%.

#### Frequency of the polymorphisms in a population of seedlings of regenerated plants

The presence of the 630-bp fragment was also studied in the progeny of regenerated plants from a separate experiment on somaclonal variation. From seeds obtained by selfing a large number of R1 plants (Van den Bulk et al. 1990) a selection was made that also included seeds of (relatively rare) diploid plants regenerated from hypocotyl tissue. Also, a small number of lines was included that carried a tissue culture-induced morphological mutation. In this experiment, only one R2 plant of each of the parent R1 lines was tested.

When the DNA of these R2 plants was compared using probe H9D9, it was found that 5 of 64 plants (8%) had the extra 630-bp fragment (Table 3). This compares well with

**Table 3** Presence of the extra 630-bp fragment in *Hind*III-restricted DNA from R2 plants, hybridized with H9D9

Ploidy-level Origin	2n			4n			Total
	Leaf	Cotyl	Hypo- cotyl	Leaf	Cotyl	Hypo- cotyl	
R2 plants <sup>a</sup>							
– <sup>b</sup>	20	15	13	1	3	7	59
+	1	1	2	0	1	0	5
Total	21	16	15	1	4	7	64

<sup>a</sup> Every R2 plant originated from a different regenerated plant

<sup>b</sup> –, fragment absent; +, fragment present

the 6–7% found in the previous experiment. In one line carrying the recessive mutation ‘entire leaf’ (Van den Bulk et al. 1990; present in heterozygous form in the R1 parent, since some R2 plants showed the mutation but others did not), the polymorphism was found in one progeny plant that did not show the mutation.

Remarkably, no polymorphisms were present in tetraploid hypocotyl-derived lines. However, three plants per tetraploid parent must be tested to reduce the chance of not detecting a polymorphism that was transmitted from one of the four chromatids of each selfed parent to 5% or less. Therefore, the results leave the possibility open ( $P>0.05$ ) that 2 of the 7 tetraploid hypocotyl-derived R2 lines did produce progeny that contained the band. The frequencies of the fragment among the three explant sources in this experiment, or between diploid and tetraploid plants, were too low to draw conclusions.

## Discussion

In this paper, the effect of tissue culture on DNA methylation in tomato was studied using five repetitive probes (Table 1). In leaf DNA, the methylation of cytosines, as detected by the isoschizomers *Msp*I and *Hpa*II, varied widely, from no detectable methylation to complete methylation at nearly all sites screened. Only small differences in methylation were found between callus and leaf DNA (Fig. 1).

When these probes were used on DNA samples digested with *Hind*III, polymorphisms were observed between control leaf and callus DNA with two probes, H9D9 and H9H6. The differences between the two tissues were most pronounced with the former probe: while in leaf DNA most of the signal was present in a 530-bp fragment, in callus DNA most of the signal was in the high-molecular-weight fraction, and an extra fragment of 630 bp appeared. This extra fragment was also present in a number of R1 and R2 plants. Several measures have been taken to ensure complete digestion of the DNA with *Hind*III. Therefore, we assume that at these *Hind*III sites, differential methylation is being measured. It is known that *Hind*III is inhibited by

methylated adenine and cytosine (Huang et al. 1982; McClelland and Nelson 1985).

The notion that *Hind*III sites in plants can be methylated is supported by other reports. First, although the simplified view is that 5-methyl-cytosine is only present in CG dinucleotides and CNG di- and tri-nucleotides (in plants), Gruenbaum et al. (1981) showed for wheat-germ DNA that, while mC was present in >80% of the CG, CAG and CTG sequences, it was also present in 19% each of CA and CT, along with 7% of CC. This correlates well with the specificity of DNA methylases (Sano et al. 1983; Adams et al. 1990). In *Petunia*, mC was found outside (G and CNG sequences (Meyer et al. 1994). Second, other modified bases have also been found in plant cells, including N<sup>6</sup>-methyladenine (Ngernprasirtsiri et al. 1988, 1989; Vanyushin and Kirnos 1988; Dhar et al. 1990, Kobayashi et al. 1990). Whether the methylation that is seen here at *Hind*III sites is adenosine methylation or cytosine methylation in sequences other than CG or CNG, remains to be determined.

Some reports also point at the methylation of *Hind*III sites in plants. Müller et al. (1990) observed polymorphisms among rice plants regenerated from a single callus when the DNA was restricted with *Hind*III. The plants that had shown polymorphisms also showed digestion with *Dpn*I, indicating the presence of N<sup>6</sup>-methyladenine in these plants. Brown et al. (1991) found such large differences among maize callus cultures after a *Hind*III digest (other enzymes were not used) that the authors wondered how it was possible that the majority of the regenerated plants had ‘reverted’ to the parental genotype. Such reversion is what would be expected if the enzyme detected callus-specific methylation, i.e. methylation of specific bases that are not usually methylated in leaf DNA.

In the present study, most of the signal from probe H9D9 in callus DNA was in the high-molecular-weight fraction, indicating methylation of both *Hind*III sites flanking the 530-bp fragment in a large number of copies. The occurrence of a 630-bp fragment may be caused by methylation of one *Hind*III site flanking the 530-bp fragment, with another, unmethylated, *Hind*III site approximately 100 bp away. This extra fragment of 630 bp was also present in 13% of the regenerated plants (Table 2). In these plants, the fragment may therefore be considered as a remnant of the *de novo* methylation that occurs during the callus phase of tissue culture, which failed to be removed in some regenerated plants. In regenerated maize plants (Kaeppeler and Phillips 1993) only decreases in methylation were detected. In barley double-haploids, however, both increase and decrease of methylation was found (Duvaux et al. 1993), while in carrot root explants *de novo* methylation took place in the presence of auxin (Arnholdt-Schmidt 1993).

The methylation at CCGG sites around the probe sequences is not changed. At first sight, this may seem to indicate that the methylation at the *Hind*III sites is changed very specifically. However, if a different methylase is involved, the change in methylation need not be subtle or local.

Few studies have determined the inheritance of methylation changes induced by tissue culture. In maize, methylation changes were 100% inherited in two generations of progeny plants (Kaeppler and Phillips 1993). In tobacco, 5-azacytidine-induced hypomethylation was maintained during plant regeneration (Koukalová et al. 1994). In our study, the 630-bp fragment is only present in 40–50% of the offspring of those R1 plants that contained the band. Therefore, the fragment was only partly inherited by the progeny lines. In all, 6–8% of the second generation offspring lines contained the extra band.

Can the polymorphism found here among regenerants from tissue culture, and their progeny, be used to increase our knowledge about the factors influencing the occurrence of these changes in tissue culture? At first sight, a 6–8% variation seems quite high; however, the plants and lines showing the polymorphism were scattered over different explant sources, different periods of tissue culture before appearance of the shoots, and different ploidy levels. With respect to the origin of the explant, 9 of 10 polymorphisms in the R1 plants were found in tetraploid shoots regenerated on hypocotyl explants (Table 2). The hypocotyl regenerants, which were all tetraploid, were among the last shoots to be transferred from the explants to the rooting medium: after 12 weeks of culture compared to 9 weeks for the majority of shoots from cotyledon and leaf explants. However, this may not be taken as a longer period of undifferentiated cell division, since the first development of leaf-like structures on the hypocotyl explants had been visible at an earlier stage than on the explants from other sources. Only the outgrowth of shoots from these structures was very slow, possibly related to the fact that they were tetraploid.

In the second experiment with R2 plants (Table 3), the R1 shoots had been taken from the explants after 6 weeks and after 12 weeks of culture (Van den Bulk et al. 1990). The R2 seeds used here were evenly taken from the 6- and 12-weeks groups, but 4 of the 5 lines showing a polymorphism (Table 3) were derived from the 6-weeks group. The results therefore do not indicate that longer periods of tissue culture induce more variation in DNA methylation among regenerant shoots. Also Van den Bulk et al. (1990), using the same material, did not find any effect of the length of the tissue-culture period on morphological mutations.

What does this mean for the phenomenon of variation induced by tissue culture? Although the frequency of fragment polymorphisms may be high compared to frequency of structural changes in the DNA [which was (over)estimated for morphological mutations, using the same material, to be  $1.2 \times 10^{-4}$ ; Van den Bulk et al. 1990], it is still low when the factors that may influence the frequency are studied. In order to gain more insight into causes and consequences, it will therefore be necessary to study the relationship between DNA methylation and changes induced during tissue culture in specific cases with a very limited number of variables. For this, those processes that show a high frequency of specific change(s) may be the most promising; for instance, the relationship between DNA methylation and vitrification.

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