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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 (Kaeppler 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 (Kaeppler 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% (Messeguer et al. 1991).
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 (Kaeppler and Phillips 1993). In tomato, protoplasts contained 20.1% mC, which was comparable to young seedling tissues and is among the lowest percentage found (Messeguer 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 (Kaeppler 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 cal- lus-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°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°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°C and with 16 h day light (Phillips fluorescent TL type 50 W 84RF, 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 μM IAA, and 4.6 μM 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 μM of 1-naphthaleenacetic acid, and 4 μM 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°C until DNA extraction.

DNA extraction and digestion

DNA was extracted from calli and seedling organs according to Del-laporta 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 U/μg 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-blot ted overnight onto Hybond N+ (Amersham).

Probes

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