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Osmotin-regulated reserve accumulation and germination in genetically transformed tea somatic embryos: a step towards regulation of stress tolerance and seed recalcitrance[1]

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Abstract

The osmotin gene was introduced into tea [*Camellia sinensis* (L.) O. Kuntze] somatic embryos at the globular stage of development via microprojectile bombardment, to determine the effects of this stress-tolerance-related gene on the accumulation of storage reserves and acquisition of desiccation tolerance during embryo maturation. Changes in total soluble sugars, starch and proteins in response to osmotin were estimated in the stably transformed embryos, using standard biochemical and histochemical methods, after the globular embryos had matured to the heart stage of development. These changes also were compared with two types of controls, comprising (1) untransformed embryos and (2) embryos that were bombarded without osmotin DNA. The control embryos showed poor reserve accumulation and negligible germination but, in contrast, the embryos bombarded with the osmotin gene showed a several-fold increase in storage reserves, normal maturation and 57–63% germination. When these transformed embryos were desiccated to 15.5% moisture content, only 40% germinated, although a further increase in the accumulation of storage reserves occurred. In contrast, the control embryos became necrotic and failed to germinate. This study demonstrates the use of somatic embryogenesis as a tool for understanding seed recalcitrance in tea, and suggests that transformation with stress-tolerance genes can overcome the developmental problems associated with recalcitrant seeds.

Keywords: *Camellia sinensis*, desiccation tolerance, germination, maturation, osmotin, recalcitrant seeds, reserve accumulation, somatic embryogenesis, tea

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Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is an important commercial crop (Bhattacharya and Ahuja, 2003). The popularity of tea has increased further due to its nutritive and medicinal properties (Jankun *et al.*, 1997) and, as a consequence, the demand in terms of both quantity and quality has also increased. Although crop improvement for yield and cup quality is an important requirement for the tea industry, improvement of the tea crop is limited largely by recalcitrant seed behaviour. In this regard, biotechnological approaches have the potential to overcome these constraints, and somatic embryogenesis is an important tool for understanding the mechanisms of *in vitro* embryo development. However, maturation of tea somatic embryos is abnormal generally, resulting in poor and precocious germination (Mondal *et al.*, 2000). Besides a low conversion frequency into plantlets, tea somatic embryos are also sensitive to stresses, such as chilling and desiccation (Mondal *et al.*, 2000, 2002), similar to their zygotic counterparts (Sharma *et al.*, 2004). Lack of reserve accumulation during embryo maturation and the absence of subsequent mobilization were reported to be the major causes of these problems (Mondal *et al.*, 2002). However, addition of abscisic acid (ABA) to heart- or maturation-stage somatic embryos triggered the accumulation of storage reserves, equivalent to those in maturing zygotic embryos (Sharma *et al.*, 2004). Although ABA promoted storage reserve accumulation, the percent germination of the somatic embryos (Sharma *et al.*, 2004) was lower than values reported earlier (Mondal *et al.*, 2002), and was not equivalent to the germination capacity of zygotic embryos (Bhattacharya *et al.*, 2002). This led us to believe that other important events govern the cellular adaptation to stress (as reviewed by Ingram and Bartels, 1996).

Tea embryos are incapable of producing sufficient amounts of osmolytes required for cellular adjustment and, hence, are sensitive to stress (Sharma, 2003).

Thus, besides providing valuable insights into the process of recalcitrant zygotic embryo development in tea, it was hypothesized that transfer of a stress-tolerance gene into the somatic embryos would be more effective in regulating the processes related to embryo maturation and their subsequent germination than only addition of ABA. The present paper aims to investigate the influence of osmotin, a stress-related gene, on the regulation of the maturation process of recalcitrant somatic embryos of tea. The osmotin gene encodes a cationic protein that accounts for about 10–12% of total cellular proteins. While ABA regulates the synthesis of osmotin, its accumulation is affected also by water stress and consequent cellular adjustment (Singh *et al.*, 1987).

Materials and methods

Plant material

Green fruits were collected from field-grown, mature bushes of *Camellia sinensis* (L.) O. Kuntze at the Tea Experimental Farm, Banuri, Institute of Himalayan Bioresource Technology, Palampur, India (1290 m a.s.l., 32°N and 76°E). The seeds obtained from these fruits were soaked overnight and were subsequently de-coated to yield cotyledon explants. Somatic embryos were induced on slices of these cotyledon explants, as described earlier by Sood *et al.* (1993) and Sharma *et al.* (2004). Synchronous secondary somatic embryos were obtained from the primary somatic embryos by culturing the latter on embryo development medium (EDM) in 9.0-cm Petri plates, following the method of Mondal *et al.* (2001). These secondary somatic embryos were maintained in culture laboratory conditions of 25 ± 2°C and cool fluorescent light of 52 $\mu mol\, m^{-2}\, s^{-1}$ with a 12-h photoperiod. Globular-stage embryos were used for the transformation experiments, whereas the transformed and control embryos that had developed into the heart stage were used as the material for all the other experiments. Two types of control, consisting of (1) untransformed embryos and (2) embryos that were bombarded without osmotin DNA, were used in all experiments. In a separate set, the control and transformed embryos were also desiccated under high relative humidity for 60 min. For each experiment, five replicates of 50 embryos were transferred to 9.0-cm Petri plates, and each experiment was repeated three times.

Transformation of globular somatic embryos with the osmotin gene via microprojectile bombardment

Plasmid

Three types of naked vector DNA were used. The ultrapure naked genomic DNA from *Micrococcus luteus* (Sigma, India) and the pRT99gus construct, harbouring *gus* and neomycin phosphotransferase genes (Töpfer *et al.*, 1988) under the control of a CaMV35S promoter and terminator, were used as controls. The construct harbouring the osmotin and neomycin phosphotransferase genes under the control of the CaMV35S promoter and terminator (Fig. 1), in the GV2260 strain of *Agrobacterium* in a binary vector form, was used for transforming the somatic embryos. Plasmid DNA harbouring osmotin was isolated from *Agrobacterium tumefaciens* culture, whereas the controls were isolated from *Escherichia coli* cultures. All of these were grown overnight at 37°C in liquid Luria Broth supplemented with 50 $mg\, l^{-1}$ kanamycin monosulphate using a Plasmid Extraction Kit (Qiagen, India), according to the manufacturer's manual.

Microprojectile bombardment of somatic embryos

The plasmid harbouring the osmotin gene and the above-mentioned two controls were used for microprojectile bombardment. The un-bombarded somatic embryos and embryos bombarded with naked gold particles served as additional negative controls. Gold particles (1 μm; Bio-Rad, Hercules, California, USA) were coated with 10 μg of plasmid DNA and used for

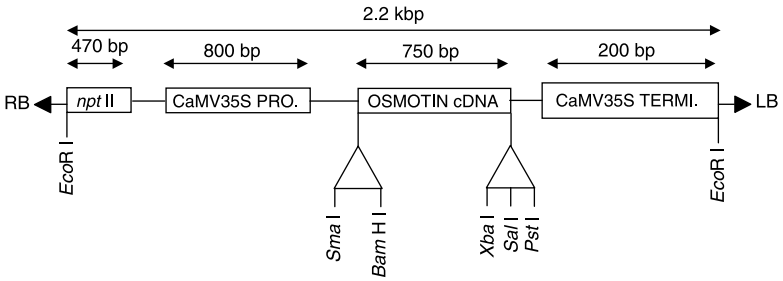

Figure 1. Linear plasmid map indicating the localization of the osmotin gene and *npt* II under the control of CaMV35S promoter (pro.), terminator (termi.) and sites for different restriction enzymes. Source: NaCl-tolerant cell suspensions of *Nicotiana tabacum* 'White Burley'.

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particle bombardment of the globular somatic embryos, using the Biolistic PDS-1000/He System (Bio-Rad). The somatic embryos placed on agar-solidified medium were bombarded using a burst pressure of 1100 psi and 9 cm target distance (Sandal *et al.*, 2004). The bombarded embryos were cultured on EDM in the dark for 2 d under culture laboratory conditions, and finally subcultured onto selection medium, containing 200 $\mu g\,ml^{-1}$ kanamycin for further proliferation and germination.

PCR analysis of the putative transformants

Six weeks after transfer to the antibiotic selection medium, total genomic DNA was extracted from 500 mg of randomly selected somatic embryos from the controls and putative transformants harbouring the osmotin gene, following the method of Doyle and Doyle (1990). Respective plasmid DNAs were used as the controls. The protocol of Mondal *et al.* (2001) was employed for the polymerase chain reaction (PCR) amplification of pRT99gus, using both *gus* and *npt* II specific primers. For osmotin, a total of about 500 ng of genomic DNA was used as the template in a 50 µl PCR mix containing 200 µM of each of dATP, dCTP, dGTP and dTTp, 4 nmol of each primer, 2.5 U *Taq* DNA polymerase and 5 µl 10 × *Taq* polymerase buffer. For amplification, the DNA was subjected to 35 cycles, comprising denaturation at 94°C for 1 min, amplification at 55°C for 1 min and annealing at 72°C for 2 min. Finally, the reaction was subjected to a further extension cycle for 7 min at 72°C, using a programmable Stratagene Robocycler Gradient 40 (La Jolla, California, USA) with the fastest available transitions between temperatures. The primer sets (Bangalore Gene I, Bangalore, India) were expected to give a product of 700 bp from the internal sequence of the osmotin gene and were as follows:

Forward: 5′−CTGCCACTATCGAGGTCCGA−3′

Reverse: 5′−CCACTTCATCACTTCCAGGC−3′.

Finally, the amplification products were separated on a 1.4% agarose gel and photographed under a UV transilluminator (Fotodyne MP-St equipped with MP4 Polaroid Instant Camera System; Photodyne Inc., Hartland, Wisconsin, USA). A molecular marker of 500 bp (Bangalore Gene I, India) was used.

Southern hybridization

In order to confirm the integration of the different transgenes into the somatic embryos, both the transformed and control embryos were subjected to Southern hybridization. Total genomic DNA was extracted from 500 mg tissue of controls and putative transformants, following the method of Doyle and Doyle (1990). Twenty micrograms of total genomic DNA from the transformants, the control embryos and the plasmid DNA were digested overnight with restriction enzymes. In order to confirm the stable integration of *gus* and *npt* II genes in the case of pRT99gus transformed somatic embryos, *Hin*dIII (Invitrogen, Life Technologies, India, Pvt. Ltd.) was used in one set and *Pst* I in a second set of digestions at 37°C. In the case of osmotin-transformed somatic embryos, *Eco* RI (Invitrogen, Life Technologies) was used in one set and the flanking restriction enzymes, *Bam* HI and *Xba* I, in a second set of double digestions at 37°C. The digested DNA of each line was separated on a 1.2% agarose gel prepared in 1 × Tris–borate–EDTA (TBE) buffer (Sambrook *et al.*, 1989), and fragments were transferred from the agarose gel to a nylon membrane (Hybond-N, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and cross-linked to the membrane by UV, using a Stratalinker (1200 Joules 100; Stratagene). The DNA fixed on the membrane was hybridized with the osmotin gene probe that was labelled by digoxigenin (DIG) DNA labelling, following the manufacturer's protocol (Roche Diagnostics, Indianapolis, Indiana, USA). For probing *gus* and *npt* II in the case of pRT99gus, digested transformants, controls and plasmids were probed with a *Hin*dIII fragment and *Pst* I fragments, respectively. Two types of probes were made in the case of osmotin, one with the *Eco* RI fragment and one with *Bam* HI and *Xba* I fragments. These were eluted from a 1.2% gel and were used to hybridize separately the respective digests. Thereafter, an alkaline phosphatase conjugate of an anti-DIG antibody was bound to the hybridized probe. Hybridizing bands were detected by visualizing with NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt), a chromogenic substrate that produces a blue-coloured signal directly on the membrane at the site of probe–target hybrids.

Biochemical studies

Biochemical and histochemical assays were conducted using un-bombarded somatic embryos, embryos bombarded with pRT99gus, naked genomic DNA from *Micrococcus luteus* and empty gold particles, as well as in the osmotin-transformed somatic embryos. Embryos were bombarded at the globular stage, and the biochemical assays were performed after they had attained the heart stage on the embryo development medium. For each experiment, three replicates were analysed, and each experiment was repeated three times. For the estimation of total soluble sugars, the anthrone method, as outlined by McCready *et al.* (1950), was used. Fresh tissue samples of the transformed somatic embryos and the four types of control embryos at heart stage were extracted in 80% ethanol. Starch was measured as released glucose

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using the anthrone reagent, following hydrolysis of the extracted powders with perchloric acid (Adams *et al.*, 1980).

Protein content was determined in all the controls and the transformed somatic embryos at heart stage of development (100 mg ml^{-1} fresh tissue) by homogenization in chilled 50 mM Tris–HCl (pH 7.0), 10% sodium dodecyl sulphate (SDS), 1% insoluble polyvinyl pyrrolidone (w/v), 10% glycerol (v/v) and 70 mM 2-β-mercaptoethanol. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were used for the determination of total protein, following the method of Bradford (1976). The standard curve was prepared with bovine serum albumin.

Histochemical studies

Fresh heart-stage transformed, somatic embryos were harvested, and 50 μm-thick hand sections were cut with a sharp blade and subjected to histochemical assays by staining them as described below. The stained sections were transferred to glycerin and examined under a Nikon Labophot microscope and photo-micrographed at 10 ×, 40 × and 100 × magnifications. Starch grains were visualized by a weak solution of iodine (0.3 g iodine and 1.5 g potassium iodide in 100 ml water). The appearance of a deep blue colour indicated the presence of starch. Fats were visualized by staining for 20 min with a Sudan III solution (0.5 g dye in 100 ml of 70% alcohol). This was followed by a quick wash with 50% alcohol, and the red-coloured stain indicated the presence of fats in the sections. Proteins were detected with 1% (w/v) aqueous potassium ferricyanide. Sections were stained with this solution, followed by a wash with 50% alcohol, and finally stained with a small drop of ferric chloride. Appearance of dark blue spots indicated the presence of proteins. The above-mentioned biochemical and histochemical assays were also conducted with the somatic embryos that were desiccated by drying the embryos in open Petri dishes under a laminar hood cabinet for 60 min.

Germination and moisture content

The effect of desiccation on germination was assessed by drying transformed heart-shaped embryos in open Petri dishes under the laminar hood cabinet for 60 min, following the method of Mondal (1999). Emergence of the radicle, and greening and elongation of the hypocotyl, coupled with the opening of cotyledonary leaves, characterized normal germination (Mondal *et al.*, 2002). Three replicates with ten embryos in each were sampled, and the untransformed embryos served as the control. The moisture content of the transformed and untransformed embryos, before and after desiccation (as described above), was recorded on fresh weight basis, following the method of ISTA (1985).

Results

Microprojectile bombardment of globular somatic embryos

Only 7–10% of the total globular somatic embryos bombarded with the osmotin gene produced secondary somatic embryos on EDM supplemented with 200 μg ml^{-1} kanamycin to yield 'heart-stage' somatic embryos. These kanamycin-resistant embryos proliferated further into secondary somatic embryos on fresh EDM supplemented with kanamycin, and germinated to the extent of 57–63% within 6–8 weeks. When placed on kanamycin, the untransformed embryos and all the controls underwent drying and died, leaving only the ones that were transformed with pRT99gus. The embryos that were placed on EDM without kanamycin remained fresh and produced secondary embryos but did not germinate.

Molecular analysis of putative transformants

Introduction of the osmotin and *npt* II genes into the respective genomes of somatic embryos of tea was confirmed after primary analysis of the transformants through PCR, which yielded the expected band of 700 bp for osmotin in the transformed embryos (Fig. 2), and a band of 470 bp of *npt* II for pRT99gus (Fig. 3) in control embryos. However, no amplification was observed in the un-bombarded embryos (controls, Figs 2–3), in those bombarded with *M. luteus* DNA and in the embryos bombarded with empty gold particles (data not shown). Integration of the osmotin, *npt* II and *gus* genes in the respective genomes of the transformed and control somatic embryos was further confirmed by Southern hybridization. For control somatic embryos that were transformed with pRT99gus, the presence of linear >6.7 kb *Hin*dIII and *Pst* I fragments, containing the *npt* II and *gus* genes, confirmed stable integration (Fig. 4A, B). A single, large internal transgene fragment of >2.1 kb was obtained from somatic embryos transformed with the osmotin gene, and it showed homology with the hybridization signals of the *Eco*RI digested plasmid DNA when probed with the entire plasmid (Fig. 5A). However, when the genomic DNA of osmotin-transformed somatic embryos and the plasmid DNA (osmotin) were probed with the PCR-amplified osmotin, after double digestion with *Bam* HI and *Xba* I, a small single internal transgene fragment of

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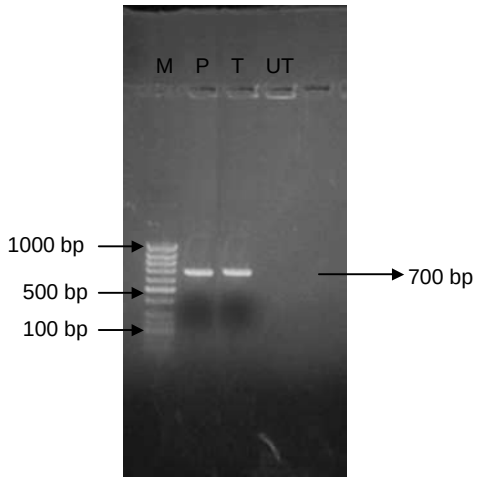

Figure 2. Polymerase chain reaction (PCR) amplification of a 700 bp internal fragment of the osmotin gene. P, plasmid; T, putatively transformed tea somatic embryos; UT, untransformed tea somatic embryos; M, molecular markers.

680 bp was generated, which was absent in sample prepared from untransformed embryos (Fig. 5B) and the other controls (data not shown). No hybridization signals were observed for any of the four controls.

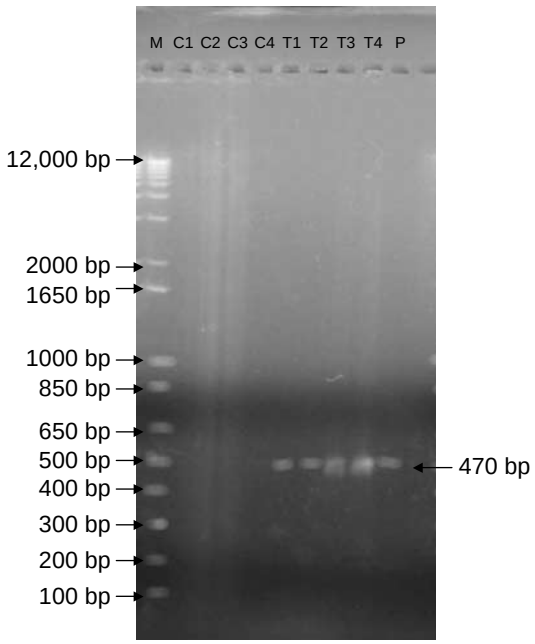

Figure 3. Polymerase chain reaction (PCR) amplification of a 470 bp internal fragment of the *npt* II gene from pRT99gus. P, plasmid; C1–C4, untransformed tea somatic embryos; T1–T4, putatively transformed tea somatic embryos; and M, molecular markers.

Biochemical studies

Starch, total soluble sugars (TSS) and proteins increased in the putatively transformed somatic embryos with the osmotin gene (Table 1). The TSS in the putatively transformed somatic embryos was much higher {72.0 mg [g fresh weight (fw)]$^{-1}$} compared to the untransformed control [13.2 mg (g fw)$^{-1}$]. Starch [60 mg (g fw)$^{-1}$] and total soluble proteins [2.1 mg (g fw)$^{-1}$] were substantially higher compared to the untransformed control [3.6 mg (g fw)$^{-1}$ and 0.6 mg (g fw)$^{-1}$, respectively]. The biochemical constituents of the embryos bombarded without DNA were similar to those of the untransformed control (Table 1). The total soluble protein content [mg (g fw)$^{-1}$] in embryos transformed with osmotin, pRT99gus and control were 4.68, 3.19 and 0.824 mg (g fw)$^{-1}$ before desiccation, and 6.26, 4.02 and 1.52 mg (g fw)$^{-1}$ after desiccation, respectively (Fig. 6). After desiccation, the somatic embryos transformed with osmotin showed an additional increase in TSS [84.0 mg (g fw)$^{-1}$] and starch [81.9 mg (g fw)$^{-1}$], compared to the control embryos or transformed, but not desiccated, somatic embryos (Table 1).

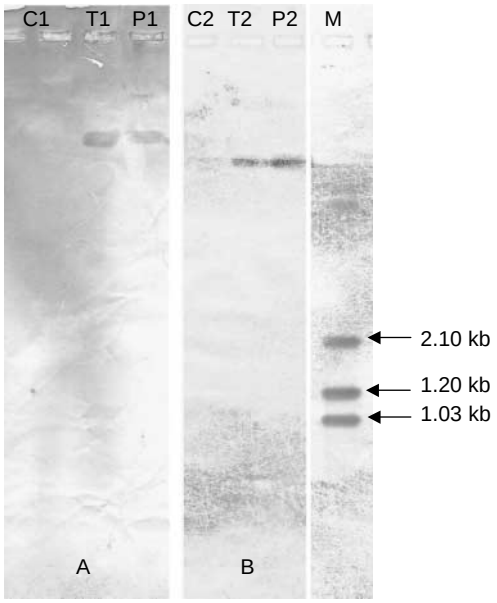

Figure 4. Southern blot analysis of somatic embryos of tea transformed with pRT99gus showing the hybridization signals, confirming the stable integration of (A) *gus* and (B) *npt* II, when large digoxigenin (DIG)-labelled *Hin*dIII and *Pst* I fragments were used as probes, respectively; C1, C2, untransformed somatic embryos; T1, T2, putatively transformed somatic embryos; P1, P2, plasmid harbouring osmotin; M, molecular markers.

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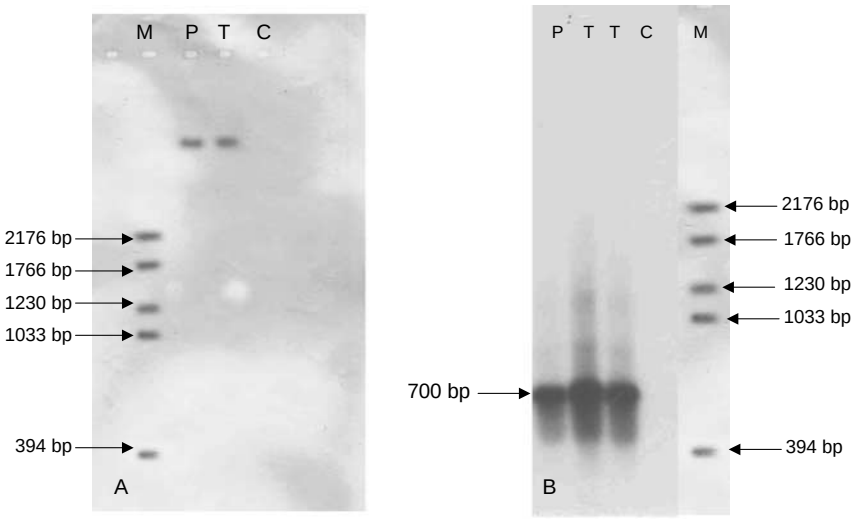

Figure 5. Southern blot analysis of osmotin-transformed somatic embryos of tea, showing the expected hybridization signals when (A) a digoxigenin (DIG)-labelled *Eco* RI fragment from the osmotin construct was used as a probe; (B) DIG-labelled osmotin fragments from *Xba* I and *Bam* H1 sites were used as probes. C, untransformed somatic embryos; T, putatively transformed somatic embryos; P, plasmid harbouring osmotin; M, molecular markers.

Histochemical studies

The untransformed control tested negative for both starch and protein (Fig. 7A, I), compared to the osmotin-transformed heart-stage somatic embryos, where abundant deposition of starch and proteins was observed (Fig. 7B, J). The number of oil droplets, which were detectable in each of the four types of treatments (Fig. 7E–H), increased by several fold in the embryos transformed with osmotin gene (Fig. 7F). All these features were more pronounced in the putatively transformed somatic embryos after desiccation (Fig. 7D, H, L). The starch, lipids and proteins in pRT99gus-transformed somatic embryos (Fig. 7C, G, K) showed a pattern similar to that of the untransformed control, despite the presence of the *npt* II gene.

Germination and moisture content

Germination of the somatic embryos bombarded with osmotin gene was 57–63%. However, when they were subjected to desiccation stress for 60 min, germination declined to 40%. Germination of the untransformed control and those bombarded without DNA ranged between 1–3% and 3–5%, respectively. Germination of embryos transformed with pRT99gus was 5%; after desiccation, pRT99gus-transformed embryos did not germinate.

The moisture content (MC) of the untransformed control was 30.2%, and the pRT99gus and osmotin transformants had moisture contents of 20.2% and 38.2%, respectively, prior to desiccation. After desiccation MCs were 6.0%, 7.2% and 15.5%, respectively (Table 2).

Table 1. Changes in biochemical constituents [mg (g fresh weight)$^{-1}$] in heart-stage somatic embryos of tea in response to bombardment of the globular somatic embryos with and without DNA from a plasmid harbouring an osmotin gene

	Untransformed control (not bombarded)	Bombarded with osmotin DNA	Desiccated after bombardment with osmotin DNA	Bombarded without DNA
Total soluble sugars	13.2	72.0	84.0	15.7
Starch	3.6	60.0	81.9	2.8
Proteins	0.6	2.1	4.5	0.6
Germination (%)	1–3	57–63	40.0	3–5

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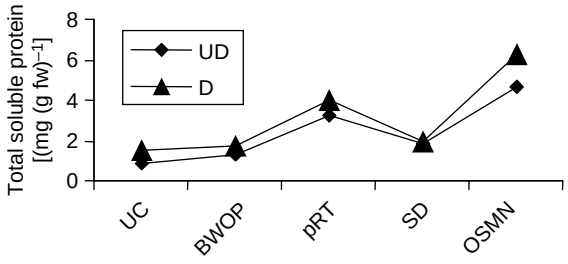

Figure 6. Protein content of somatic embryos of tea at the heart stage. UC, untransformed control; BWOP, bombarded without plasmid; pRT, bombarded with pRT99gus; SD, bombarded with naked DNA; OSMN, bombarded with osmotin; UD, not desiccated; D, desiccated.

Discussion

The objective of the present study was to investigate the effect of delivering the osmotin gene to somatic embryos of tea at an early stage of development, and then observe its effects during the maturation of somatic embryos. Transformation with the osmotin gene has been reported to increase tolerance to different biotic and abiotic stresses, including desiccation, in many plants (Grover *et al.*, 2001). Somatic embryos of tea exhibit a typical recalcitrant seed character; they do not mature normally (Sharma *et al.*, 2004) and generally show a high frequency of precocious germination (Vieitez, 1995). Like their zygotic counterparts (Bhattacharya *et al.*, 2002), the somatic embryos of tea are also sensitive to desiccation and other stresses (Mondal *et al.*, 2000, 2002), and are incapable of undergoing cellular adjustments (Sharma, 2003). The cationic protein osmotin is known to bring about cellular adjustment through accumulation of osmolytes/osmoprotectants and a subsequent increase in tolerance to different stresses, including desiccation (Pla *et al.*, 1998). Thus, it was thought that introduction of this gene into developing somatic embryos would facilitate the process of normal embryo maturation, comprising accumulation of storage reserves and acquisition of desiccation tolerance (McKersie *et al.*, 1990). The present study showed that this was indeed true for tea somatic embryos. Whereas the control embryos failed to germinate after desiccation and died, 40% of the transformed embryos germinated. This indicated that the problems of tea somatic embryogenesis could be partially alleviated by genetic transformation with a stress-tolerance gene. PCR amplification confirmed that the delivery of the osmotin gene into globular

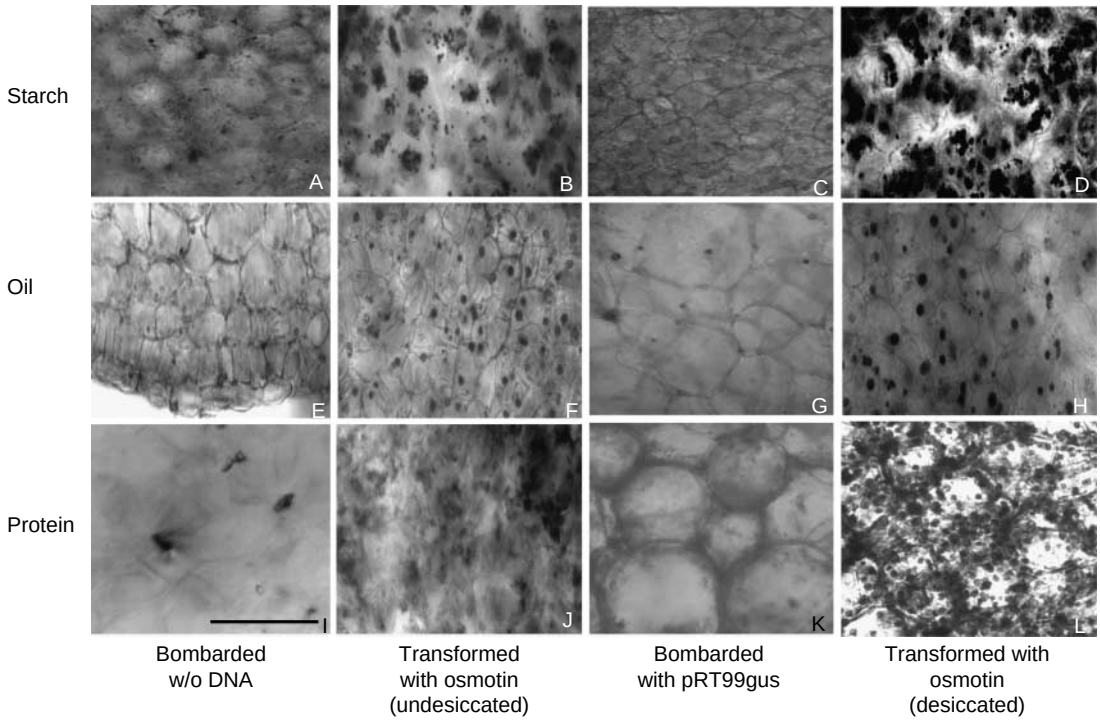

Figure 7. Histochemistry of heart-stage embryos of tea showing: (A) starch, (E) oil droplets and (I) proteins after bombardment without DNA; (B) starch, (F) oil droplets and (J) proteins in transformants with osmotin; (C) starch, (G) oil droplets and (K) proteins in somatic embryos bombarded with pRT99gus used as a control; and (D) starch, (H) oil droplets and (L) proteins in desiccated osmotin transformants (bar = 100 μm).

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Table 2. Moisture contents (MC) of heart-stage tea somatic embryos after various treatments, before and after desiccation

Treatments	Moisture content before desiccation (%)	Moisture content after desiccation (%)
Untransformed control	30.2	6.0
Transformed with pRT99	20.2	7.2
Transformed with osmotin	38.2	15.5
Transformed with naked DNA	18.6	7.0
Bombarded without plasmid	16.6	5.4

somatic embryos had taken place, and its stable integration into the genome was confirmed at the heart stage by Southern hybridization. The positive influence of the osmotin gene was apparent from the high percentage (57–63%) of normal somatic embryo germination after transformation, as well as by the increase in the storage reserves at the maturation stage (Table 1, Fig. 7). The ability to germinate normally to 40% after desiccation, compared to no germination or no accumulation of storage reserves in the desiccated untransformed somatic embryos, further confirmed that osmotin was responsible for the acquisition of a certain degree of desiccation tolerance in the somatic embryos of tea. These responses were the consequence of transformation with the osmotin gene, and not due to the transformation process *per se*, because the embryos that were bombarded without the osmotin gene did not exhibit reserve accumulation, desiccation tolerance or germination >1–5%. In our previous study, ABA also induced normal maturation of the tea somatic embryos, followed by 50% normal germination, compared to very low germination in the untreated control (Sharma *et al.*, 2004). However, tolerance of the ABA-treated embryos to desiccation stress was not examined.

Although the germination of tea somatic embryos could be improved considerably (57–63%) through genetic transformation with the osmotin gene, germination was much lower than that of their zygotic counterparts. Further improvement in germination of the somatic embryos of tea requires investigation of other regulatory cues that are provided by the surrounding maternal tissues of the zygotic embryos for normal development and germination (Kermode *et al.*, 1986). It would be pertinent to mention here that, although the zygotic embryos are equipped with the ability to germinate normally in high frequency, they are incapable of tolerating desiccation stress, and hence cannot be stored for long periods without loss of seed viability. In view of this, the present study seems important, because it not only indicates that somatic embryos can serve as an important biochemical tool for the understanding of the *in vivo* processes of zygotic embryogenesis, but also shows that engineering stress tolerance can be an important route to circumventing the problems of desiccation intolerance in recalcitrant embryos. Overcoming the problem of desiccation intolerance and enhancing long-term storage of recalcitrant embryos/seeds is an important field of research. Any solution in this direction is bound to have a direct bearing on the field of seed biology and germplasm conservation of many important plant species.

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