Stable transformation of Mesembryanthemum crystallinum (L.) with Agrobacterium rhizogenes harboring the green fluorescent protein targeted to the endoplasmic reticulum

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ABSTRACT

Stable transformation of Mesembryanthemum crystallinum L (common ice plant) with a green fluorescent protein (GFP) construct targeted to the endoplasmic reticulum was obtained. Seven and fourteen days after germination seedlings were infected with Agrobacterium rhizogenes strain ARqua1 either by direct coating of the cut radicles with bacteria growing on solid medium or by immersion of the cut surface in bacterial suspension at different optical densities. Both methods of infection resulted in production of GFP-positive roots with a frequency ranging from 6 to 20% according to the age of the explants and the application procedure. The green fluorescing roots displayed the typical hairy root phenotype and were easily maintained in liquid medium without growth regulators for over 2 years. Stable expression of the transgene in the roots was confirmed by polymerase chain reaction (PCR), immunoblotting and the capacity of roots to grow and produce callus on kanamycin-enriched medium. Nineteen endogenous cytokinins were determined in transgenic and non-transformed roots. The results revealed significantly lower levels of the free bases of isopentenyladenine, dihydrozeatin, cis- and trans-zeatin, as well as a conspicuous decline in concentrations of the corresponding nucleosides and most nucleotides in transgenic roots compared to the wild type. Comparison of the cytokinin profiles in transgenic and non-transformed roots suggested that transformation by A. rhizogenes disturbed cytokinin metabolism during the early steps of biosynthesis. Calli obtained from transformed roots were GFP-positive and remained non-regenerative or displayed high rhizogenic potential depending on the auxin/cytokinin ratio in the medium. Calli and callus-derived roots showed a strong GFP signal for over 2 years.

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; cZ, cis-zeatin; cZ9G, cis-zeatin-9-glucoside; cZOG, cis-zeatin-O-glucoside; cZR, cis-zeatin riboside; cZR5 MP, cis-zeatin riboside-5′-monophosphate; DHZ, dihydrozeatin; DHZ9G, dihydrozeatin-9-glucoside; DHZOG, dihydrozeatin-O-glucoside; DHZRS MP, dihydrozeatin riboside-5′-monophosphate; DHZR, dihydrozeatin riboside; GFP, Green fluorescent protein gene; GPP, green fluorescent protein protein; IAA, indole-3-acetic acid; iP, isopentenyladenine; iP5G, isopentenyladenine-9-glucoside; iP8R, isopen-tenyladenosine; iP8R-SP, isopen-tenyladenosine-5′-monophosphate; Kinetin, 6-furfurylaminopurine; MS, Murashige and Skoog medium (1962); NAA, 1-naphthaleneacetic acid; OD600, optical density at 600 nm; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PGR, plant growth regulator; SDS, sodium dodecyl sulfate; tZ, trans-zeatin; tZ9G, trans-zeatin-9-glucoside; tZR, trans-zeatin riboside; tZR5 MP, trans-zeatin riboside-5′-monophosphate.

1 These authors contributed equally to this study.

Introduction

Mesembryanthemum crystallinum L. (common ice plant) is a slow-growing, annual halophyte that displays facultative Crassulacean acid metabolism (CAM) (Adams et al., 1998). The specific features of ice plant biology, such as extreme tolerance to high salinity, stress-inducible switching from C3 photosynthesis to CAM as well as accumulation of betalains under enhanced irradiation make this organism a useful experimental model to study molecular aspects of plant responses to different environmental stimuli (Bohnert and Cushman, 2000). Recently, the common ice plant has also emerged as a suitable plant for studying mechanisms underlying organogenesis, particularly the role of hydrogen per-
oxide and polar auxin transport in root development (Libik et al., 2005; Konieczny et al., 2009). However, the ice plant has been regarded a plant species recalcitrant to molecular and genetic approaches. Although hairy-root and transgenic callus cultures from M. crystallinum have been established after transformation with Agrobacterium rhizogenes (Andolfatto et al., 1994) and A. tumefaciens (Ishimaru, 1999), there is still a need to improve the efficiency of transformation to make it useful for functional genomic studies and targeted gene transfer (Bohnhart and Cushman, 2000).

The green fluorescent protein (GFP) from the jellyfish Aequora victoria has proven to be a powerful tool in plant genetic transformation studies (reviewed by Hraška et al., 2006). Its main advantage over other visual markers of transformation is that it allows non-invasive observation of the transgene in real time in living plants. The usefulness of GFP for aiding improvements of Agrobacterium-mediated transformations has been reported for several plant species, e.g. carrot (Baranski et al., 2006), pear (Yancheva et al., 2006) and potato (Rakosy-Tican et al., 2007). To date, differently modified forms of the GFP gene have been constructed for enhanced expression, altered spectral properties and different compartmentalization in plant cell organelles. The mgfps-ER construct allows GFP targeting to the endoplasmic reticulum (ER), causing only very little detrimental effect on metabolism and differentiation (Haseollof et al., 1997). Its usefulness for monitoring the efficiency of transformation in different dicot and monocot species has been reported previously (reviewed by Hraška et al., 2006). Thus far, nothing is known about the efficiency and performance of GFP-reporter constructs in the common ice plant.

In several species, both shoot and somatic embryo formation from hairy roots failed (Christey, 2001). One of the reasons for these difficulties is hormonal imbalance of transgenic tissue due to transfer and expression in host bacterial genes directly involved in plant hormone metabolism (Tanaka et al., 2001). In hairy roots of common ice plant, Andolfatto et al. (1994) reported a changed level of auxin compared to the wild type and regeneration of transgenic plants was not obtained. Cytokinins, together with auxin, level of auxin compared to the wild type and regeneration of transgenic plants of common ice plant. Quantitative data from transformation experiments were supplemented with analysis of endogenous content of different cytokinins in hairy roots and roots derived from non-transformed plants.

### Materials and methods

#### Plant material

The procedures for plant growth and seed sterilization were as described previously (Libik et al., 2005). Sterile seeds were germinated on 9 cm Petri dishes filled with MS basal medium (Murashige and Skoog, 1962) with 30 g L\(^{-1}\) sucrose and 5 g L\(^{-1}\) agar (Difco, USA), pH 5.7. The seeds were incubated at 25/20 °C, a 16/8 h (light/dark) photoperiod and 120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) light intensity (cool fluorescent tubes) in phytotron chambers. After 7 or 14 days of germination, the apical part of seedling primary roots (about 3 mm from the tip) was cut off and the remaining part of the seedling was used in transformation experiments.

#### Bacterial strain and plasmids

Agrobacterium rhizogenes strain ARqua1 (Quandt et al., 1993) harboring the binary plasmid vector pCB302 containing 35S::pGFP5:ER expression cassette was used in the transformation experiments. The plasmid carried the green fluorescent protein (GFP) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the selectable marker, neomycin phosphotransferase II (nptII), under the control of the nopaline synthase (nos) promoter.

#### Bacterial cultures

Bacterial cultures were grown from frozen (−80 °C) stocks on solid YEB medium containing 5 g L\(^{-1}\) beef extract, 3 g L\(^{-1}\) peptone, 1 g L\(^{-1}\) yeast extract, 0.17 g L\(^{-1}\), MgSO\(_4\), 5 g L\(^{-1}\) sucrose, 15 g L\(^{-1}\)agar, 25 mg L\(^{-1}\) kanamycin and 100 mg L\(^{-1}\) streptomycin, pH 7.2. All chemicals were purchased from Sigma (Germany) except for the agar, which was obtained from Difco (Detroit, USA). Cultures of bacteria were maintained for 2 days at 25 °C in the dark before they were used for transformation.

#### Transformation procedure

Two different procedures of infection were used. In the first method, the cut surface of the seedling primary root was directly coated with bacteria by dragging it on the surface of plate containing Agrobacterium colonies. In the second procedure, the cut surface of the seedling was immersed for about 30 s in liquid bacterial cultures of four different OD\(_{600}\) (0.5, 1.0, 2.0 or 3.0) and subsequently blotted on sterile filter paper. Liquid cultures of bacteria were prepared as follows: bacteria from a liquid culture in YEB medium were spun down (5000 × g for 10 min) and the pellet was resuspended to yield the desired OD\(_{600}\) in liquid MS medium free of plant growth regulators (PGRs) (pH 5.7) but with 30 g L\(^{-1}\) sucrose. For each experiment, 109–120 seedlings were used (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Infection procedure</th>
<th>No. of infected explants</th>
<th>7-days-old seedlings producing GFP positive roots (%)</th>
<th>Mean no. of GFP positive roots</th>
<th>No. of infected explants</th>
<th>14-days-old seedlings producing GFP positive roots (%)</th>
<th>Mean no. of positive roots</th>
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<tr>
<td>Dragging</td>
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<tr>
<td>OD(_{600}) = 0.5</td>
<td>114</td>
<td>9a</td>
<td>10.4 ± 2.1a</td>
<td>117</td>
<td>18b</td>
<td>11.4 ± 3.1a</td>
</tr>
<tr>
<td>OD(_{600}) = 1.0</td>
<td>120</td>
<td>6a</td>
<td>9.6 ± 1.2a</td>
<td>120</td>
<td>20b</td>
<td>10.7 ± 2.8a</td>
</tr>
<tr>
<td>OD(_{600}) = 2.0</td>
<td>112</td>
<td>0</td>
<td>11.3 ± 0.1a</td>
<td>120</td>
<td>20b</td>
<td>10.1 ± 1.8a</td>
</tr>
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</table>

Values with the same letter within a column do not significantly differ by Tukey’s range test (P ≤ 0.05).
Culture of transformed roots and calli

After infection with *Agrobacterium*, 5–6 seedlings were placed side-by-side on a single Petri dish (9 cm in diameter) containing solid MS (30 g L⁻¹ sucrose, 8 mg L⁻¹ Difco agar, pH 5.7) free of PGRs. Cultures were maintained for 48 h followed by subculture on the medium of the same composition but with addition of 250 mg L⁻¹ cefotaxime (Sigma, Germany) to suppress bacterial growth. After 6 weeks on medium with antibiotic, single roots showing green fluorescence were dissected from mother seedlings under the fluorescence stereomicroscope and transferred to solid PGR-free MS for 14 days followed by batch subculture in 250 ml Erlenmeyer flasks containing liquid PGR-free MS (pH 5.7) with 30 g L⁻¹ sucrose. Cultures were maintained on a rotary shaker (150 rpm) and subcultured in fresh MS every 4 weeks. To induce calli, single hairy roots from liquid culture were transferred onto solidified MS (30 g L⁻¹ sucrose, 8 mg L⁻¹ Difco agar, pH 5.7) containing amin (IAA, NAA or 2,4-D at 0.2, 0.5, 1, 2, 4 mg L⁻¹) or cytokinin (kinetin or iP at 0.5, 1, 2 mg L⁻¹) alone or in combinations (IAA, NAA or 2,4-D at 1, 2 or 4 mg L⁻¹ plus kinetin or iP at 0.2, 0.5, 1 or 2 mg L⁻¹). For each treatment, 50 hairy roots were used. Additionally, 25 randomly chosen hairy roots derived from liquid culture were tested for their ability to produce callus with permanent kanamycin selection on solid MS (as described above) with 0.5 mg L⁻¹ kinetin, 1 mg L⁻¹ 2,4-D and 25 or 50 mg L⁻¹ of antibiotic. Roots from non-transformed plants growing in soil in the phytotron chamber (conditions as for seed germination) were used as a control. Once induced, calli were subcultured every 4 weeks on media of the same composition. All cultures were kept in a growth chamber under the conditions described for seed germination.

Microscopic GFP detection

Transgenic roots and calli were identified by visualizing GFP expression by a fluorescence stereomicroscope (Leica DMi4000B) equipped with GFP2 – 510 nm long pass emission filter with a 480 ± 40 nm excitation filter and GFP3 – 525 ± 50 nm emission filter with a 470 ± 40 nm excitation filter. Microscopic observations of GFP fluorescence in single roots and callus cells were performed with a fluorescence microscope Axiosvert 135 (Zeiss) equipped with a 450–490 nm excitation and a 520 nm wide pass emission filter. Imaging of ER in transgenic hairy roots and in root-derived callus was performed with an Olympus FV1000 confocal laser scanning microscope (CLSM) by using a 60× water-immersion objective. GFP fluorescence was imaged using excitation with the 488-nm line of the multi-line argon laser. Images were processed by image processing software (Photoshop, Adobe Systems Inc., Mountain View, CA, USA).

Molecular confirmation of transformation

After 1 year of culture in liquid MS, the transgenic roots exhibiting green fluorescence were further analyzed for stable integration of mgfp5-ER construct by PCR amplification and the presence of mGFP5-ER protein by immunoblotting. Genomic DNA was isolated from green fluorescent roots using the DNeasy plant kit (Qiagen). A 850 bp fragment representing the coding region of mgfp5-ER was amplified using the upstream 5’-CACATTAGGAAGCATGCC-3’ and polyA uppers 5’-cttacttgcctgctac-3’. Amplification was performed using a Peltier Thermal Cycler (MJ Research) at 94 °C for 90 s, followed by 30 cycles at 94 °C for 60 s, 59.8 °C for 120 s and 72 °C for 180 s. PCR products were visualized after electrophoresis on a 1% agarose gel stained with ethidium bromide under UV irradiation. The plasmid pBIN-GFP5-ER was used as a positive control, while genomic DNA from stems and leaves of *Mesembryanthemum crystallinum* after 2 months of transformation was used as negative control.

Immunoblotting was performed using GFP-specific antibody. After extraction using the method of *Hurkman and Tanaka* (1986), total proteins were separated by SDS-PAGE on 10% acrylamide gels. Proteins were blotted to PVDF membranes (PeqLab) according to *McCubbin et al.* (1997) and then incubated sequentially with the primary mouse anti–GFP antibody (1:1000) (Roche) and the secondary anti-mouse antibody coupled to alkaline phosphatase (1: 7500) (Promega). After detection, the PVDF membranes were scanned on a HP Scan Jet 3400C (Hewlett-Packard). As negative controls, extracts from stems and leaves of *M. crystallinum* after 2 months of transformation were used. The molecular weight of protein was determined according to the PeqGold Protein-Marker IV (PeqLab).

Cytokinin measurements

Hairy roots maintained for 2 months in liquid MS (as described above) without PGRs were used to determine the effect of transformation by *A. rhizogenes* on endogenous cytokinin content. As a control, primary lateral roots (3–4 cm in length) derived from non-transformed 2-month-old plants growing in vitro in 250 ml Erlenmeyer flasks containing PGR-free MS (30 g L⁻¹ sucrose, 8 mg L⁻¹ Difco agar, pH 5.7) under the same conditions as liquid cultures of hairy roots were used. The procedure for sterilization and germination of seeds of donor plants for cytokinin assay was as described in the Plant material section. The procedure used for cytokinin purification was a modification of the method described by *Faisst et al.* (1997). Deuterium-labeled CK internal standards (Olcemim Ltd., Czech Republic) were added, each at 5 pmol per sample to check the recovery during purification and to validate the determination (*Schwartenberg et al.*, 2007). The samples were purified using an immunoaffinity chromatography (IAC) based on wide-specificity monoclonal antibodies against cytokinins (*Novák et al.*, 2003). The metabolic eluates from the IAC columns were evaporated to dryness and dissolved in 20 μL of the mobile phase and were used for quantitative analysis. The samples were analyzed by ultra-performance liquid chromatography (UPLC; Acquity UPLCTM; Waters, Milford, MA, USA) linked to a Quattro microTM API (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electro spray interface, as described recently (*Novák et al.*, 2008). Quantification was performed by multiple reaction monitoring of [M+H]+ and the appropriate product ion by Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to the appropriate labeled standard was determined and used to quantify the level of endogenous compounds in the original extract, according to the known quantity of added internal standard (*Novák et al.*, 2008).

Statistical analysis

Transformation experiments were performed using 5 Petri dishes per treatment as replicates. The experiments were repeated four times. After 6 weeks of infection with bacteria, the frequency of transformation (percentage of explants producing roots expressing GFP compared to the whole number of *Agrobacterium*-infected seedlings) as well as the mean number of GFP-positive roots produced per single explant (total number of primary lateral fluorescing roots divided by number of seedlings producing roots with GFP fluorescence in a particular culture) were determined. Cytokinin content was determined on root samples from 3 healthy non-transformed plants growing in vitro and 3 liquid cultures established from hairy roots of 3 different seedlings transformed with the use of bacterial solution at OD600 > 2.0. Measurements were done in three replicates. Differences between means were
tested by a multivariate analysis of variance and a Tukey’s range test (transformation experiments) or Student-Newman–Keule test (cytokinin analysis) using Statistica for Windows ver.8.0 (StatSoft, Inc., Tulsa, Oklahoma). Data on graphs and in Table 1 represent the means ± standard errors (SE).

Results

Hairy roots induction and culture

The roots showing bright GFP fluorescence occurred directly from the main root of seedlings after 2 weeks of inoculation with *Agrobacterium* (Fig. 1A and B). These roots were produced along with normal, nonfluorescent roots with an average number of about 10 roots per explant at the 6th week of co-culture with bacteria (Table 1). When excised from original explants and put separately onto solid MS free of PGRs, these fluorescing roots grew rapidly (Fig. 1C–F). They could be easily subcultured into PGR-free liquid medium, where they have been grown vigorously and showing a strong GFP signal for over 2 years (Fig. 1G–J). All GFP-positive roots displayed intensive branching, plagiotropic growth and numerous long root hairs. The intensity of GFP fluorescence was similar throughout the roots. However, in some cases the strongest signal was observed at the root tip and in the central cylinder (Fig. 1I–J). Proper localization of GFP to the ER was revealed by CLSM in living root cells, showing tubular ER networks and sheets in the transformed root epidermal cells (Fig. 1K). In addition to the ER network and nuclear envelope, bright fluorescent spindle-like ER bodies, well described in *Brassicaceae* species including *Arabidopsis* (Haseloff et al., 1997), were also visualized in the ice plant by using mgfp5-ER construct (Fig. 1K star).

Both PCR and immunoblot analyses confirmed the presence and stable integration of the GFP transgene by revealing the predicted band of 850 bp for mgfp5-ER after PCR amplification and the predicted 29 kDa band for GFP5-ER on immunoblots from all samples of transgenic fluorescing roots. On the other hand, no mgfp5-ER transgene and no GFP5-ER protein were detected by PCR and immunoblot in stems and leaves representing a negative control from the same seedlings (Fig. 2A and B).

The method of infection, as well as the age of explants, strongly affected the percentage of seedlings producing hairy roots but had no effect on the number of roots produced per single explant (Table 1). The coating of the cut radicles with bacteria growing on solid medium provided a 2-fold higher frequency of transformation when 14-day-old explants were used (18 and 9%, respectively). The infection by immersion of seedlings in a suspension of AQua1 (with mgfp5-ER) at a concentration of OD₆₀₀ = 0.5 failed to induce hairy roots but did not disturb the normal development of seedlings, which grew into plants with well-differentiated root systems. Increasing the bacterial density up to OD₆₀₀ = 2.0 increased the efficiency of transformation about 2-fold for 14-day-old seedlings compared to 7-day-old ones (12 vs. 6% and 20 vs. 9% for AQua1 suspensions used for infection at 1.0 and 2.0 at OD₆₀₀, respectively (Table 1). The seedlings infected with the highest concentration of bacteria (OD₆₀₀ = 3.0) tended to be overgrown by Agrobacterium and were unable to produce roots at all. Within 10–15 days after inoculation on MS with cefotaxime, these explants became necrotic and died.

Callus culture

When transferred from liquid culture to solid MS containing cytokinin (kinetin or iP at 0.5–5 mg L⁻¹) or IAA (0.2–4 mg L⁻¹) alone, the hairy roots turned brown and died, but they formed numerous short lateral roots with clear GFP fluorescence when maintained in the presence of 2,4-D or NAA at 0.2 or 0.5 mg L⁻¹. The growth of these lateral roots was limited and they never exceeded 1 cm in length (Fig. 3A and B). After 2 weeks on media supplemented with 1, 2 or 4 mg L⁻¹ of 2,4-D or NAA hairy roots produced calli with a frequency of 100%. Addition of cytokinin (ip or kinetin) at 0.2 or 0.5 mg L⁻¹ along with 2,4-D or NAA did not influence the frequency of callogenesis but strongly stimulated callus growth (Fig. 3C). No differences were observed between cytokinin type and the course of callogenesis. Concentrations of cytokinin higher than 0.5 mg L⁻¹ (1 or 2 mg L⁻¹) completely inhibited both 2,4-D and NAA-induced
callus production. After 4 weeks of culture on medium containing 2 mg L\(^{-1}\) NAA together with 0.5 mg L\(^{-1}\) kinetin or iP, most of the calli (90 and 100%, respectively) started to produce numerous roots (36.4 and 34.6 roots per callus, respectively) (Fig. 3E). Occasionally (12% of calli; 5.4 roots per explant), rhizogenesis was also observed from callus tissue maintained on medium supplemented with 4 mg L\(^{-1}\) NAA and 0.5 mg L\(^{-1}\) iP (Fig. 3F). Calli cultured on media containing other combinations of plant hormones remained non-regenerative throughout the culture period. All calli induced in the experiments, as well as callus-derived roots, regularly showed strong GFP fluorescence for over 2 years (Fig. 3G and H). Detailed microscopic observations revealed that single cells of these calli exhibited the same levels of GFP fluorescence (Fig. 3I–K). In callus-derived roots the strongest fluorescence was again found at the root tip and/or in the central part of the organs (Fig. 3G). The rhizogenic potential of calli was maintained for over 2 years. Addition of 25 or 50 mg L\(^{-1}\) kanamycin to the medium containing 0.5 mg L\(^{-1}\) kinetin and 1 mg L\(^{-1}\) 2,4-D did not influence the ability of the green fluorescent roots to form calli: all explanted roots produced vigorous and GFP-positive calli within 2–3 weeks after explantation. In contrast, control non-transformed roots died within 10 days after exposure to kanamycin.

Cytokinin content

The cytokinins detected in control roots were: the free bases, \(tZ\), \(cZ\), DHZ and iP; their ribosides (\(iZR\), \(cZR\), DHZR, iP) and 5'-nucleotides (\(iZR5\) MP, \(cZR5\) MP, DHZR5' MP, iP5 MP) as well as both O- and N-glucosides of \(tZ\)-, \(cZ\)- and DHZ-type cytokinin (\(cZOG\), \(tZOG\), DHZOG and \(cZ9G\), \(tZ9G\), DHZ9G, respectively) (Fig. 4A–D). The most abundant cytokinins were \(cZ\)-type with \(cZMP\) and \(cZ\) having the highest overall concentration, followed by \(tZ\)-type, iP-type and DHZ-type. Except for \(cZ\)-type, where N-glucosconjugate (cZ9G) accumulated to a concentration higher than its O-glucoside (cZOG),

Fig. 2. Molecular confirmation of transformation of 14-days old seedlings of \(M.\ crystallinum\) with \(A.\ rhizogenes\) ARqua1 infected by direct coating of cut surface of seedling main root with bacteria. (A) PCR analysis with specific GFP-ER primers. Lanes: L – DNA marker, 1 – genomic DNA from green fluorescing roots of \(M.\ crystallinum\); 2 – negative control with genomic DNA from stems and leaves of infected seedlings of \(M.\ crystallinum\); 3 – positive control with plasmid \(pBIN-GFP5-ER\). (B) Immunoblot detection of GFP-ER protein showing single band at appr.29 kDa using GFP-specific antibody. Lanes: L – protein marker; 1 – protein extract from stably transformed green fluorescing roots of \(M.\ crystallinum\); 2 – negative control with protein extract from stems and leaves of infected seedlings of \(M.\ crystallinum\).

Fig. 3. Production and culture of green fluorescing callus after infection of 14-days-old seedlings of \(M.\ crystallinum\) with \(A.\ rhizogenes\) strain ARqua1 at OD\(_{600}\) = 2.0. (A, C–F) Images in white light; (B, G, H) images from fluorescence stereomicroscope using GFP2 or GFP3 filters; (I, J, K) epifluorescence microscopic images. (A, B) Single green fluorescing root maintained for 10 days on solid MS medium containing NAA at 0.2 mg L\(^{-1}\). (C, D) Production of non-regenerative callus from single green fluorescing root after 14 days (C) and 4 weeks (D) on MS medium containing 1 mg L\(^{-1}\), 2,4-D and 0.5 mg L\(^{-1}\) kinetin. (E, F) Effect of two different NAA concentrations: 2 mg L\(^{-1}\) (E) and 4 mg L\(^{-1}\) (F) on rhizogenic potential of calli after 6 weeks of induction on media containing 0.5 mg L\(^{-1}\) iP. Arrowheads point to roots. (G, H) Calli from media supplemented with 2 mg L\(^{-1}\) NAA and 0.5 mg L\(^{-1}\) iP (G) and 1 mg L\(^{-1}\), 2,4-D and 0.5 mg L\(^{-1}\) 2,4-D and 0.5 mg L\(^{-1}\) kinetin (H) showing strong GFP fluorescence after 1 year of induction. (I–K) Individual cells of callus maintained for 2 years on MS containing 1 mg L\(^{-1}\), 2,4-D and 0.5 mg L\(^{-1}\) kinetin. Arrowheads point to nucleus. Bar represents 0.1 mm for A, B; 5 mm for C–H and 50 μM for I–K.
the remaining isoprenoid cytokinins showed several times higher concentrations of O-glucosylated forms than the corresponding N-glucosides. No N9-glucouonjugates (iP9G) of iP-type cytokinins were detected. In hairy roots, the concentrations of almost all cytokinins studied were significantly lower than in wild type roots (Fig. 4A–D). The exceptions were DHZR5'MP and DHZR concentrations, which were about 3- and 2.5-fold higher in transgenic roots than in controls, respectively, and DHZ and its N-glucoside (DHZ2G) concentrations which remained close to those detected in non-transformed roots. Compared to controls, the most pronounced decline was observed in metabolism of iP-type cytokinins, so that iP5'MP and its free base iP, were not detected at all, while iPR was present in only trace amounts in hairy roots. Transgenic roots also displayed approximately 8-fold lower content of Z and Z' nucleotides (cZ5'MP and tZ5'MP) and about a 10-fold lower concentration of cZR compared to wild type roots. In the case of Z-type, the reduction in corresponding riboside content was less conspicuous than for cZ-type, changing only about 2-fold. In hairy roots, the O-glucoside conjugates of all isoprenoid cytokinins (tZOG, cZOG, DHZOG) were strongly reduced compared to the control material (Fig. 4A–D).

Discussion

The results revealed the capability to transform the common ice plant with GFP as a reporter gene. The bacterium used in the experiments was A. rhizogenes strain ARqua1 bearing the binary plasmid vector pCB302-mgfp5-ER. The ARqua1 strain has been shown to efficiently generate hairy roots in cultures of Vicia hirsuta and Medicago truncatula (Quandt et al., 1993; Boisson-Dernier et al., 2001) and, more recently, in Populus tremuloides (Cseke et al., 2007). In the procedure applied here, the selection of transgenic roots was performed visually by using GFP fluorescence, and the presence of the transgene was confirmed by PCR and immunoblotting. Some previous reports have suggested that GFP can replace antibiotic selection (e.g. Yancheva et al., 2006; Baranski et al., 2007). In our study, single green fluorescent roots could produce GFP-positive calli when maintained on media supplemented with kanamycin. In contrast, non-fluorescing roots derived from Agrobacterium-infected seedlings of ice plant were not kanamycin-resistant and died when exposed to this antibiotic. Because the T-DNA used here for the ice plant transformation contained the NPTII gene conferring resistance to kanamycin along with GFP gene, these results support the usefulness of GFP as a means to detect and reliably select stable transformed tissues and organs in our material.

In our experiments, 14-day-old seedlings were found to be more susceptible to Agrobacterium than 7-day-old ones, which is consistent with the well-known dependency between the physiological state and the age of the target tissue and the efficiency of transformation (Karami et al., 2009). The optimal density of Agrobacterium suspension for ice plant transformation was OD₆₀₀ = 2, and a concentration lower than this caused a significant decrease, or even complete failure of transformation (Table 1). The optimal density of the bacterial inoculum determined here for the common ice plant is about two-fold higher than reported for the majority of species (Veena and Yaylor, 2007). The reason for this could be related to the bacterial strain and/or to specific features of the host tissue: the ARqua1 strain is characterized by a relatively low virulence (Quandt et al., 1993) and the ice plant is known to possess a high intrinsic resistance to bacterial diseases (Bohnert and Cushman, 2000). In our study the sectioned radicles of seedlings were also inoculated by direct coating with Agrobacterium grown on YEB medium. This simple method of infection has been routinely used in Medicago truncatula (Boisson-Dernier et al., 2001) and in the case of ice plant it resulted in frequencies of transformation comparable to those found after immersion of explants in the bacterial suspension at OD₆₀₀ = 2.0 (Table 1). Although the seedlings infected by this procedure developed normally and did not show any signs of contamination, it is important to keep in mind that this method does not allow the control of the amount of bacteria in the single inoculum. Thus, for use in a standard transformation procedure in the common ice plant, we recommend dipping the explants in a bacterial suspension of defined density.

Microscopic observations revealed that hairy root-derived calli of common ice plant exhibited similar levels of GFP fluorescence throughout the tissue, suggesting their origin entirely from the...
transformed cells. As observed in other studies, the production of chimeric calli with green fluorescence in some, but not all, cells would be a possible outcome as a result of transgene silencing in continued culture (e.g. Baranski et al., 2007; Rakosy-Tican et al., 2007). However, as shown here, the intensity of green fluorescence of calli produced from transformed roots of the common ice plant did not change, and individual callus cells exhibited similarly high, uniform levels of GFP fluorescence even after 2 years of culture, indicating high transgene stability. Although very stable GFP fluorescence was also observed in the hairy roots directly induced from seedlings of the ice plant, most of them showed differential level of GFP fluorescence, with the strongest signal within the central cylinder and at the root tip. The intensity of GFP fluorescence depends on several factors, such as the GFP gene variant and the type of promoter as well as the target tissue and the species used (Haška et al., 2006). The CaMV 35S promoter that we used in our work does not ensure uniform expression of the transgene and is suggested to favor actively dividing cells like those in meristems (Nagata et al., 1987; Baramski et al., 2007). This may partly account for the strongest GFP signal being found at the apical region of ice plant roots and along cambial zone within vascular cylinder. Strong and stable GFP fluorescence was observed within the roots of common ice plant, while the remaining parts of the seedling were non-fluorescent throughout the culture. Because the formation of fluorescing roots was accompanied by normal seedling development, the procedure described here opens the opportunity for the generation of so-called “composite plants” comprising of transgenic hairy roots growing out from a non-transformed mother seedling.

Callus tissue is often an intermediate stage in the process of producing genetically modified plants. In M. crystallinum, successful whole plant regeneration via callus was reported by both somatic embryogenesis (Cushman et al., 2000; Libik et al., 2005) and organogenesis (Meiners et al., 1991; Wang and Lüttge, 1994). Unfortunately, the use of media of similar hormonal and mineral composition to those reported in the papers mentioned above did not result in either shoot or somatic embryo formation from transgenic callus in ice plant. Root locus (rol) genes of the A. rhizogenes Ri-plasmid are necessary for hairy root formation and they are also directly involved in plant hormone metabolism (White et al., 1985; Tanaka et al., 2001). Andolfatto et al. (1994) reported that the hairy roots of the common ice plant contained elevated levels of free IAA compared to non-transformed ones. In this study, we showed that transformation by A. rhizogenes resulted in a conspicuous change in concentration of both free cytokinins (iP, Z, Z) and their glucosides and phosphate derivatives in transformed roots. According to the current model of isoprenoid cytokinin biosynthesis, the 5'-nucleotides are direct precursors of cytokinin ribosides, which in turn are converted into free base species (Sakakibara, 2006). The results of our investigation show that transformed roots displayed considerably lower amounts of Z-, Z-, and I-riboside-5'-monophosphates than non-transformed ones. It can be regarded as an indication that in common ice plant the transformation by A. rhizogenes disturbed cytokinin metabolism at the very early steps of biosynthesis. This could also account for the observed decrease in Z29G and Z29G content in transgenic roots: cytokinin N-glucosides are believed to be not cleaved by cellular and bacterial glucosidases and the decline in their content is usually ascribed to slower biosynthesis of the corresponding free base (Sakakibara, 2006).

To promote regenerative competence, exogenous PGRs supplied to the culture medium and endogenous hormones in the tissue of the primary explant must be correctly balanced (Jimenez, 2005). Our results indicated that there were extremely low cytokinin levels in hairy roots. This may be a reason for the difficulties with plant regeneration from transgenic tissue. However, simply increasing the concentration of exogenous cytokinin in the medium did not influence the morphogenic potential of transgenic ice plants. Transformation by A. rhizogenes is known to influence the metabolism of several endogenous plant hormones including auxin and abscisic acid (ABA) and that plant hormones are involved in the regulation of each other’s abundance (Christey, 2001). For example, in roots of Arabidopsis thaliana, a high level of auxin or ABA was shown to downregulate the expression of genes directly involved in synthesis of both Z-type and i-type nucleotides (Takei et al., 2004). Interestingly, an increased pool of IAA was found to accompany the decline in cytokinin content in transformed apple (Lambert et al., 1998). As noted above, elevated levels of IAA have also been observed in hairy roots of ice plant (Andolfatto et al., 1994). Thus, we cannot rule out the possibility that a decrease in cytokinin pool in the transgenic roots of the ice plant could be a secondary effect of transformation-induced disturbance in metabolism of other(s) hormone(s). It is also possible that some differences in cytokinin metabolism between non-transformed and transgenic roots may appear because transgenic roots were maintained separately in liquid medium, while the controls in our experiments were roots taken directly from in vivo plants. On the other hand, vigorous growth of isolated transgenic roots in liquid MS free from PGRs seems to confirm Agrobacterium-induced modification in the endogenous phytohormone metabolism in transgenic tissue as the isolated but non-transformed roots of ice plant could not grow on artificial medium without added PGRs (Andolfatto et al., 1994; data not shown). Further analysis is required to verify the mechanism of the effect of A. rhizogenes effect on hormonal homeostasis in the common ice plant.

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References

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