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RESEARCH PAPER

The application of the yeast *N*-acetyltransferase *MPR1* gene and the proline analogue L-azetidine-2-carboxylic acid as a selectable marker system for plant transformation

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Abstract

The yeast *N*-acetyltransferase *MPR1* gene has previously been shown to confer resistance to the toxic proline analogue azetidine-2-carboxylic acid (A2C) in yeast and transgenic tobacco. Here experiments were carried out to determine if *MPR1* and A2C can work as a selectable marker system for plant transformation. The *MPR1* gene was inserted into a binary vector under the control of the cauliflower mosaic virus 35S promoter and nopaline synthase terminator, and transformed into tobacco via the *Agrobacterium tumefaciens*-mediated leaf disc method. A2C was applied in the selection medium to select for putative transformants. PCR analysis showed that 28.4% and 66.7% of the plantlets selected by 250 μM and 300 μM A2C were positive for the *MPR1* gene, respectively. Southern and northern blot analysis and enzyme activity assay confirmed the stable gene incorporation, transcription, and translation of the *MPR1* transgene in the transgenic plants. The transgene-carrying T_1 progeny could be distinguished from the recessive progeny when grown on 400, 450, or 500 μM A2C. Examination of the metabolism of 22 transgenic plants by gas chromatography–mass spectrometry profiling did not reveal any significant changes. In conclusion, the results demonstrate that *MPR1*/A2C is a safe and efficient selection system that does not involve microbial antibiotic or herbicide resistance genes. Recent studies showed that MPR1 can protect yeast against oxidative stresses by decreasing the accumulation of the proline catabolite Δ^1-pyrroline-5-carboxylate (P5C). However, H_2O_2 treatment resulted in contradictory responses among the five transgenic lines tested. Further experiments are required to assess the response of *MPR1* transgenic plants under oxidative stress.

Key words: *N*-Acetyltransferase, L-azetidine-2-carboxylic acid (A2C), *MPR1*, proline analogues, selectable marker, tobacco (*Nicotiana tabacum* L. cv. Xanthi).

Introduction

Genetic transformation allows direct introduction of beneficial agricultural traits into crop plants such as disease and pest resistance, stress tolerance, or the production of biofuel or pharmaceutical compounds. Such changes have been demonstrated to reduce the application of harmful pesticides to the environment, improve crop productivity and land use efficiency (reviewed by Ramessar *et al.*, 2007), and can provide sustainable supplies of beneficial compounds. Unfortunately, its application in crop improvement has been hindered due to public rejection, a major reason being the use of antibiotic or herbicide resistance selectable markers for the generation of transgenic crops. Developing alternative, environment-friendly selectable marker systems, therefore, has become an important and continuing task in

the field of plant biotechnology. Approximately 50 selectable marker genes had been reported up to 2004 (reviewed by Miki and McHugh, 2004). However, their practical application in crop plants is limited and the most effective and commonly used systems remain the antibiotic kanamycin resistance (neomycin phosphotransferase, *nptII* gene), hygromycin resistance (hygromycin phosphotransferase, *hpt* gene), and herbicide phosphinothricin resistance (phosphinothricin acetyltransferase, *bar* gene) (Miki and McHugh, 2004).

The yeast *MPR1* gene (sigma 1278b gene for proline-analogue resistance) was discovered in a yeast strain Σ1278b that exhibits resistance to a toxic proline analogue, azetidine-2-carboxylic acid (A2C, Fig. 1A, Shichiri *et al.*, 2001). A2C is toxic to cells because it causes the formation of defective proteins by replacing proline in protein synthesis (Fowden, 1963). The yeast *MPR1* gene encodes an *N*-acetyltransferase that is able to convert A2C to *N*-acetyl A2C, which is no longer incorporated into proteins and therefore not toxic to cells (Fig. 1B, Shichiri *et al.*, 2001). The toxicity of A2C and the ability of *MPR1* to detoxify it suggests that they can potentially work as a selectable marker system for plant transformation. To test its applicability, *MPR1* was previously overexpressed in the model plant tobacco, and the *MPR1* transgenic plants showed resistance to A2C (Zhang *et al.*, 2004), suggesting that *MPR1* is able to function in plants and impart resistance to A2C. However, attempts to use A2C in the transformation process to select for *MPR1*-transformed transgenic tobacco were not successful, as only one out of 24 A2C-resistant plants were transformed with the *MPR1* gene (Zhang *et al.*, 2004). In this study, the aim was to optimize the *MPR1*/A2C selection system and determine if it can be used for plant transformation.

A2C is a rare imino acid that is only found in some plant species such as *Convallaria majalis* (lily of the valley) of the family *Liliaceae* (Fowden, 1956) and garden beets (Rubenstein *et al.*, 2006). It is doubtful that yeasts would encounter A2C in their natural growth environment; consequently detoxifying A2C would not be the actual function of *MPR1* in yeast. BLAST search and genomic PCR analysis found *MPR1* homologous sequences in several yeast species (Nomura and Takagi, 2004) and fungal strains (Du and Takagi, 2007), suggesting that *MPR1* originated from a common ancestor and that it might serve some physiological function. Investigating its role in these organisms can potentially lead to new discoveries of previously unknown pathways. So far *MPR1* homologous sequences have not been found in available genomic sequences of plants.

Yeast cells with disrupted *MPR* (*MPR1* and a homologous *MPR2*) genes could grow normally, indicating that *MPR* genes are not essential for growth; however, these cells were hypersensitive to oxidative stresses. Closer examination revealed that *MPR*-expressing yeasts had lower reactive oxygen species (ROS) levels when subjected to oxidative stress than the *MPR* disruptants (Nomura and Takagi, 2004; Du and Takagi, 2005, 2007). Introducing *MPR* genes into the *MPR* disruptants restored their viability under oxidative stress accompanied by lower ROS levels (Nomura and Takagi, 2004). This result leads to the postulation that MPR1 can regulate ROS levels in yeast cells and prevent ROS-induced cell death in oxidative stress conditions. A2C is structurally similar to a proline biosynthesis and catabolism intermediate, Δ^1-pyrroline-5-carboxylate (P5C; Fig. 1A), whose accumulation in cells has been reported to cause generation of ROS in human cells (Donald *et al.*, 2001) and *Arabidopsis* (Miller *et al.*, 2009), and cell death (Hermann *et al.*, 2000; Donald *et al.*, 2001; Deuschle *et al.*, 2004). It is thus hypothesized that P5C, or more probably its equilibrium compound glutamate-γ-semialdehyde (GSA; Fig. 1A), is the cellular substrate for MPR1, and that MPR1 can reduce excess P5C/GSA levels by acetylation, and in turn prevent the generation of ROS and ROS-induced cell damage (Nomura and Takagi, 2004). *In vitro* enzyme assays using purified MPR1 enzyme confirmed that MPR1 can carry out an acetylation reaction with acetyl-CoA and P5C as substrates

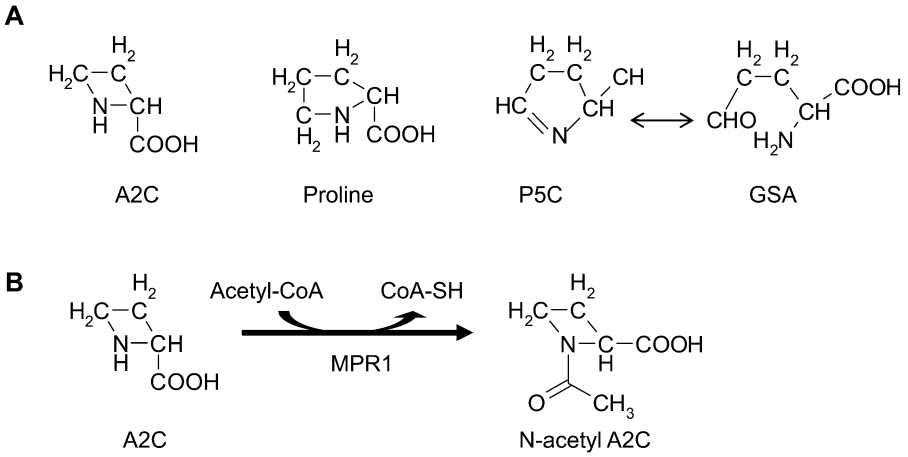

Fig. 1. (A) Structure comparison of proline, its catabolism intermediates Δ^1-pyrroline-5-carboxylate (P5C) and glutamate-γ-semialdehyde (GSA), and the analogue azetidine-2-carboxylic acid (A2C). (B) MPR1 is an acetyltransferase which can detoxify A2C by converting it to *N*-acetyl A2C (Shichiri *et al.*, 2001).

at neutral pH (Nomura and Takagi, 2004). The detailed biochemical steps involving MPR1 are currently under investigation (H. Takagi, personal communication).

The objective of this study is to determine the potential of *MPR1* and A2C as a selectable marker system for plant transformation. In the model plant tobacco, its efficiency in selecting transformed cells as well as the segregated transgenic progeny was demonstrated. The transgene expression and metabolism of the transgenic plants were characterized. Whether MPR1 confers the same protective effect in plants under oxidative stress was also assessed.

Materials and methods

Construction of the transformation vector

A promoter–polylinker–terminator backbone was first constructed in pUC18. The double (2×) cauliflower mosaic virus (CaMV) 35S promoter was excised from pCAMBIA1305.2 (CAMBIA, Canberra, Australia) by *Bst*XI and *Xho*I digestion, then ligated into pBluescript II SK$^+$ (Stratagene, La Jolla, CA, USA) in order to obtain the *Sac*I and *Kpn*I overhangs at the 5′ and 3′ end, respectively. The resultant promoter fragment was released again from pBluescript II by *Sac*I and *Kpn*I digestion and subsequently cloned into pUC18 at the corresponding sites. The *NOS* terminator was PCR amplified (FideliTaq PCR Master Mix, USB, Cleveland, OH, USA) from the binary vector pBI121 (Clontech, Palo Alto, CA, USA) with added *Pst*I at the 5′ end and *Hin*dIII at the 3′ end with primer *Pst*I-*NOS*^{t}-5′ and *NOS*^{t}-*Hin*dIII-3′ (Table 1), and inserted into pUC18 at *Pst*I and *Hin*dIII sites. After sequence verification, the completed 2× CaMV 35S promoter–pUC18 polylinker–*NOS* terminator backbone was released by *Hin*dIII and *Eco*RI digestion and cloned into the binary vector pBI121 in place of the β-glucuronidase (*GUS*) expression cassette. This engineered binary vector backbone is designated pBIDN. The 690 bp full-length *MPR1* cDNA (GenBank: AB031349) along with the 70 bp upstream and 333 bp downstream non-coding sequence was amplified by PCR from plasmid pMH1 (Zhang *et al.*, 2004) with primer *MPR1-Bam*HI-5′ and *MPR1 Sal*I-3′ (Table 1) with FideliTaq. The PCR product was cloned into pGem-T easy vector (Promega, Madison, WI, USA) and verified by sequencing before being inserted in the sense direction into pBIDN at *Bam*HI and *Sal*I sites to generate the *MPR1*-expressing binary vector pBIDN-*MPR1* (Fig. 2). pBIDN-*MPR1* was introduced into *Agrobacterium tumefaciens* strain EHA105 via electroporation.

Plant growth

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) sterile shoot cultures were initiated from seed and maintained on MS agar medium (MS salts and vitamins, 3% sucrose, 8 g l^{-1} agar) (Murashige and Skoog, 1962) in Magenta boxes (Magenta, Chicago, IL, USA). Seed was surface-sterilized in 20% (v/v) Clorox (5.25% Na hypochlorite) for 20 min and rinsed three times with sterile water before being sown on the medium. For the progeny seed segregation test, the seeds were grown on MS agar medium containing A2C (Sigma, St Louis, MO, USA) or 250 mg l^{-1} (429 μM) kanamycin (Agri-Bio, Miami, FL, USA) in 100×25 mm Petri dishes. A2C stock solution (50 mM) and medium was prepared fresh before use. Suspension cell cultures were initiated from leaves of sterile shoot cultures of wild-type and *MPR1* transgenic lines on solidified MX medium (MS with 1.8 μM 2,4-dichlorophenoxyacetic acid) and then maintained in 50 ml of MX liquid by weekly subculture in 125 ml flasks on a gyratory shaker as described in Widholm (1971). Plant growth conditions were 28 °C with a diurnal cycle of 16 h light/8 h darkness and a light intensity of 150 μmol photons m^{-2} s^{-1}.

Transformation and selection

Transformation was carried out based on the *A. tumefaciens*-mediated leaf disc method described by Horsch *et al.* (1985). Leaf segments were co-cultivated with *A. tumefaciens* strain EHA105 harbouring the binary vector pBIDN-*MPR1* (Fig. 2) followed by regeneration under the selection pressure of either kanamycin (100 mg l^{-1} or 172 μM) or A2C at the indicated concentrations. Timentin (400 mg l^{-1}, Agri-Bio) was included in the regeneration/selection medium in the early process until *Agrobacterium* had been eliminated. Regenerated shoots were excised and transferred to MS agar medium containing the same concentration of kanamycin or A2C as the regeneration medium to allow rooting. Rooted plantlets were checked by PCR for the presence of the *MPR1* transgene, and further analysed by Southern and northern blot hybridization. Confirmed transgenic lines were transplanted to pots and grown in a greenhouse to obtain self-pollinated T_1 progeny seed.

PCR screening of putative transgenic lines

One young leaf was harvested from each of the putative transgenic plantlets or T_1 progeny seedlings, immediately frozen in liquid nitrogen, freeze-dried (Flexi-Dry™ MP, FTS® Systems, Stone Ridge, NY, USA), and homogenized with a Fast Prep FP120 Cell Disrupter (Savant Instruments, Holbrook, NY, USA). Genomic DNA was isolated using the CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson, 1980). PCR analysis was carried out with *Taq* DNA polymerase (New England BioLabs, Beverly, MA, USA) with primers *MPR1-Bam*HI5′ and *MPR1-Sal*I3′ (Table 1), which amplify the full-length 1093 bp of the *MPR1* gene.

Southern and northern blot analyses

Approximately 1 g of leaf tissues were harvested from the wild type as well as PCR-positive transgenic tobacco shoot cultures, freeze-dried, and homogenized as described above. Genomic DNA was isolated with the CTAB method, followed by *Bam*HI restriction digestion and agarose gel (0.8%) electrophoresis. Total RNA was extracted using the TRIZOL Reagent (Invitrogen, Carlsbad,

Table 1. Primer sequences used for PCR

Primer	Sequence (5′–3′)	Added restriction site (underlined)
*Pst*I-*NOS*^{t}-5′	ATCTGCAGGATCGTTCAA ACATTTGGC	*Pst*I
NOS^t-*Hin*dIII-3′	AGAAGCTTCCGATCTAGTAAC	*Hin*dIII
*MPR1-Bam*HI-5′	ATGGATCCCGAATGCTTTACTCATATAACGG	*Bam*HI
*MPR1-Sal*I-3′	ATGTCGACGTTAACGTTAAGCCCAAAAATTCA	*Sal*I
MPR1-59F	TTTTTCAACCGTTAGCCGAC	–
MPR1-443R	TTCTGACCTCTATGGGCACC	–

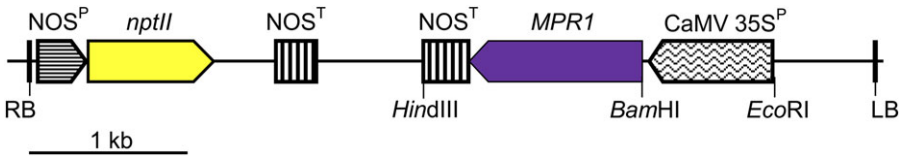

Fig. 2. Schematic map of the 5.4 kb T-DNA region of the binary vector pBIDN-*MPR1*, which was engineered from the commercial binary vector pBI121 by replacing the *GUS* with the *MPR1* expression cassette between *Hin*dIII and *Eco*RI. CaMV 35S^P, duplicated cauliflower mosaic virus 35S promoter; *MPR1*, 1093 bp *MPR1* gene comprising the full-length *MPR1* cDNA (690 bp) and part of its 5′- and 3′-non-coding sequence; *NOS*^{T}, nopaline synthase terminator; *nptII*, kanamycin resistance gene; *NOS*^{P}, nopaline synthase promoter; LB and RB, left and right border. Arrows indicate the direction of transcription.

CA, USA) and separated on a 1.2% agarose–formaldehyde gel. DNA or RNA was transferred from the gel to a nylon membrane (Amersham Hybond-XL, GE Healthcare, Piscataway, NJ, USA). The 384 bp *MPR1* probe DNA was PCR generated from pMH1 with primers *MPR1*-59F and *MPR1*-443R (Table 1) and purified from an agarose gel. The membranes were hybridized with [α-^{32}P]dCTP-labelled probe DNA (Sequenase™ Random Primer Labeling Kit, USB Corporation, Cleveland, OH, USA) at 65 °C overnight, and washed according to the manufacturer's manual. Hybridized signals were exposed to an X-ray film (HyBlot CL™ Autoradiography Film, Denville Scientific Inc., Metuchen, NJ, USA) at –80 °C with intensifying screens.

MPR1 enzyme activity assay

MPR1 enzyme activity was measured as the formation rate of 5-thio-2-nitrobenzoic acid (TNB) which results from the reaction of CoA-SH with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Schichiri *et al.*, 2001; Nomura and Takagi, 2004; Zhang *et al.*, 2004). Fully expanded young leaves from shoot cultures, or 6-day-old suspension cell cultures were collected and homogenized with a pre-chilled mortar and pestle in 2 vols of ice-cold extraction buffer (100 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and protease inhibitor (cocktail set VI, Calbiochem, Gibbstown, NJ, USA) as recommended by the manufacturer. Following centrifugation at 15000 *g*, 4 °C for 20 min, the supernatant was desalted with an Econo-Pac® 10 DG column (Bio-Rad Laboratories, Hercules, CA, USA) and eluted with assay buffer (50 mM TRIS-HCl, pH 8.5). The 1 ml reaction mixture was composed of the assay buffer, 100 µl of the extract, 0.1 mM acetyl-CoA (Sigma), 1 mM DTNB (Sigma), and 1 mM A2C. The formation of TNB was monitored at 412 nm by a spectrophotometer (DU Series 640, Beckman Instruments, Fullerton, CA, USA) at 30 °C for 7 min. A blank reaction without A2C was monitored separately in order to subtract the background OD_{412} increase resulting from other acetyltransferase activities present in the extract. The reaction rate was calculated using 15570 M^{-1} cm^{-1} as the extinction coefficient for TNB (Shichiri *et al.*, 2001) where one unit OD_{412} corresponds to 64.2 nmol of TNB produced in a 1 ml reaction mix. Protein concentrations were determined using the Bio-Rad protein assay reagent. The assays were repeated three times.

Determination of intracellular ROS levels and cell viability

A 15 mg aliquot of 5- or 6-day-old suspension cells were inoculated into 15 ml of MX liquid medium in 125 ml flasks followed by H_2O_2 treatment. Treated cells were collected by filtering through miracloth with suction. The addition of H_2O_2 to each flask and the subsequent harvest of cells 45 min afterwards were done at 30 s intervals to ensure equal treatment time for each sample. Intracellular ROS levels were measured according to Nomura and Takagi (2004) as the 2′,7′-dichlorofluorescin (DCF) fluorescence resulting from the oxidation of the fluorescent dye DCF diacetate (DCFDA, Sigma) by intracellular ROS. Fluorescence was read at Ex=490 nm and Em=524 nm (SpectraMax-2, Molecular Devices, Menlo Park, CA, USA). Cell viability was determined with a CellTiter 96 Non-Radioactive Cell Proliferation assay kit (Promega) which measures the cellular formation of formazan from tetrazolium dye at 570 nm with a spectrophotometer (DU Series 640).

Gas chromatography–mass spectrometry (GC-MS) analysis

Four wild-type and 22 *MPR1* transgenic tobacco shoot cultures were transplanted to soil and grown in a greenhouse. One young fully expanded leaf was collected 35 d after transplanting, frozen immediately in liquid nitrogen, and freeze-dried as described above. Ten milligrams of the homogenized leaf powders was extracted with methanol and water followed by derivatization according to Roessner *et al.* (2000). Samples (1–2 µl) were injected with a split ratio of 5:1 into the GC-MS system, which consisted of an Agilent 6890N gas chromatograph, Agilent 5973i mass selective detector, and HP 7683B autosampler (Agilent Inc., Palo Alto, CA, USA).

The column used for gas chromatography was 30 m HP-5MS (Agilent) with 0.25 mm internal diameter and 0.25 µm film thickness. The injection temperature was 250 °C. The interface and ion source were set to 250 °C and 230 °C, respectively. The oven temperature was programmed for an initial isothermal heating at 70 °C for 5 min, followed by a steady increase at a rate of 5 °C min^{-1} to 310 °C, and a final hold at 310 °C for 10 min. The flow rate of the helium carrier gas was set at 1.3 ml min^{-1}. Mass spectra were recorded in the scan range of *m*/*z* 50–800, and compared with the electron impact mass spectrum library NIST05 (NIST, Gaithersburg, MD, USA), WILEY (Palisade Corporation, NY, USA), and two custom libraries. Samples were normalized using the hentriacontanoic acid (10 mg ml^{-1}) internal standard. Data were integrated and evaluated with AMDIS (NIST) and HP Chemstation (Agilent). Statistical *t*-test was performed using Microsoft Excel 2003. Principle component analysis (PCA) was carried out on log-transformed, mean-centred and Paretto-scaled data using SIMCA-P$^+$ version 12 (Umetrics AB, Umea, Sweden).

Results

Construction of the MPR1 *binary vector for constitutive expression in plants*

A previous report has shown that the yeast *MPR1* gene could be successfully expressed in transgenic tobacco when included with its 70 bp upstream and 333 bp downstream non-coding sequences under the control of the 'super-promoter' (Zhang *et al.*, 2004). As the superpromoter was designed to drive extremely high gene expression (Ni *et al.*,

1995), which is more suitable for genes of interest than a selectable marker, in this study another binary vector was constructed using the duplicated CaMV 35S promoter which is commonly used to control selectable marker genes. This binary vector, pBIDN-*MPR1* (Fig. 2), was engineered from the commercial vector pBI121 by replacing the *GUS* gene with the *MPR1* expression cassette comprising the duplicated CaMV 35S promoter and nopaline synthase (*NOS*) terminator, and the same non-coding and the 690 bp coding sequences of *MPR1* described in Zhang *et al.* (2004). pBIDN-*MPR1* also contains the kanamycin resistance gene (*nptII*) from the pBI121 backbone, which allows comparison of the transformation and selection efficiency between the *MPR1*/A2C and the *nptII*/kanamycin system in tobacco transformation.

Determination of the optimal A2C concentration for the selection process

In order to determine the optimal A2C concentration to be applied in the selection process, wild-type tobacco leaves were cut into ~3 mm×3 mm segments and incubated on regeneration medium supplemented with A2C ranging from 100 μM to 500 μM. Inhibition of shoot regeneration could be observed at 200 μM and the inhibitory effects increased with dose (Fig. 3A). This result suggested that concentrations of ⩾200 μM could inhibit untransformed cells from regenerating and could be applied in the regeneration/selection process.

With the information provided by Fig. 3A, transformation and selection experiments were conducted testing A2C as a selective agent. After 3 d of co-cultivation with *A. tumefaciens* harbouring pBIDN-*MPR1*, tobacco leaf explants were regenerated under selection pressures of 200, 300, 400, and 500 μM A2C. Shoots were transferred to rooting medium using the same selection pressure as for the regeneration. Rooted shoots were considered putative transformants and analysed by PCR with *MPR1*-specific primers to check for the presence of the *MPR1* gene. The first experiment (data not shown) revealed that many of the plantlets obtained from 200 μM A2C selection were escapes, while higher concentrations such as 400 μM and 500 μM produced very few plantlets. Further transformation experiments were carried out using 250, 300, and 350 μM A2C in the selection process. At 350 μM shoot regeneration and rooting were poor, while reasonable numbers of PCR-positive transformants could be obtained with 250 μM and 300 μM (data not shown). It was concluded that the optimal selection concentrations for tobacco transformation should be between 250 μM and 300 μM. It was observed that the inhibitory effect of A2C decreased if the selection plates were overcrowded with plant materials. The selection efficiency could be improved by reducing the number of explants or shoots in the selection plate. Eventually 14 explants or seven shoots in one 10 cm plate, with ~1 cm space between each, were selected as the best conditions. Changing selection medium frequently, for example every 7–10 d, also improved the efficiency.

Comparison of the nptII*/kanamycin and* MPR1*/A2C selection systems for tobacco transformation*

In order to better understand the efficiency of the *MPR1*/A2C selection system, side-by-side transformation experiments were conducted comparing A2C (250 μM and 300 μM) with kanamycin (100 mg l^{-1} or 172 μM) selection. The leaf explants were co-cultivated with pBIDN-*MPR1* harbouring *A. tumefaciens* then randomly placed on kanamycin or A2C plates. The regeneration and rooting processes were photographed and are shown in Fig. 3B. Rooted shoots were harvested weekly and analysed by PCR with *MPR1*-specific primers to calculate the selection efficiency (number of PCR-positive transformants per 100 plantlets that survived the selection). As shown in Table 2, in both selection systems PCR-positive rates were the highest in the first 2 weeks then declined over time. In the case of kanamycin selection the efficiency was 81.8% in the first 2 weeks then decreased to 33.3% in the third week. Very few shoots started rooting after 3 weeks on kanamycin. In comparison, the selection efficiency of A2C in the first 2 weeks was 28.4% at 250 μM and 66.7% at 300 μM. The number of rooted plantlets increased in the fourth week at both concentrations. However, only 10% (250 μM) and 19.2% (300 μM) of the plantlets rooted in the fourth week were PCR positive, suggesting that A2C lost its effectiveness after 3 weeks. It was observed that some plantlets on A2C medium developed brown root tips (Fig. 3C). PCR analysis showed that these plantlets were escapes. However, not all escapes developed brown root tips, as only 9% did in the selection experiment with 250 μM A2C. The cause and nature of this browning are unknown, but this feature could partly assist the selection of putative transformants.

Using the same transformation procedure and technique, 63 PCR-positive transformants were obtained from 294 leaf explants with 250 μM A2C selection, with a transformation frequency of 21.4%; kanamycin selection produced 68 transformants from 14 explants, resulting in a 486.7% transformation frequency. Although A2C selection was not as efficient as kanamycin, it was efficient enough to routinely obtain reasonable numbers of transgenic tobacco lines.

Stable gene incorporation of MPR1 *into the tobacco genome*

Twenty-eight of the A2C-selected putative transformants were analysed by genomic Southern blot hybridization. Single to multiple copies of the *MPR1* transgene were found in all transgenic lines (Fig. 4), confirming the incorporation of the *MPR1* transgene into the genome. This result suggests that A2C selection was accurate in identifying transgenic cells. In conclusion, *MPR1* and A2C can be used as a selectable marker system for tobacco transformation.

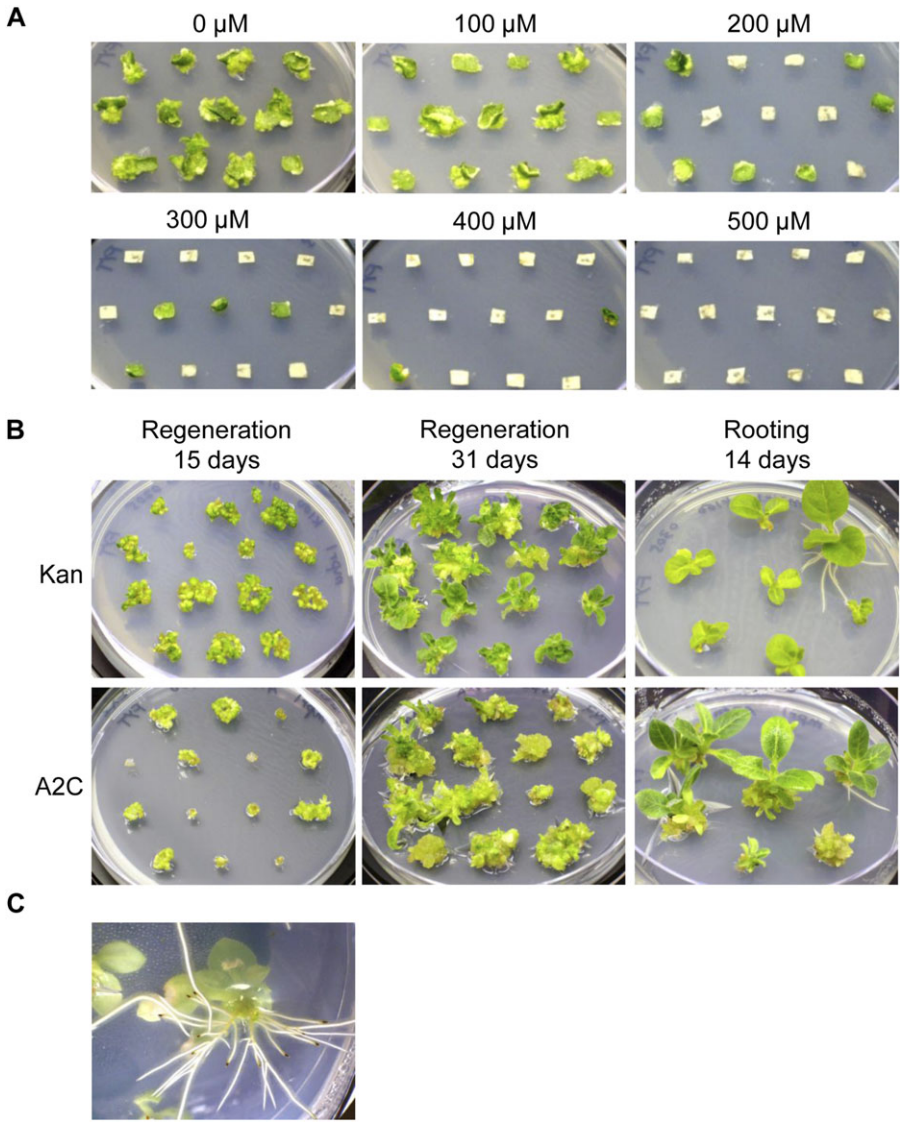

Fig. 3. (A) Effect of A2C on tobacco regeneration. The wild-type leaves were cut into 3 mm×3 mm pieces and placed on MS medium supplemented with 2 mg l^{-1} of 6-benzylaminopurine (6-BA) and a series of concentrations of A2C as indicated on top of the images. The plates were photographed 2 weeks after inoculation. (B) Tobacco transformation with pBIDN-*MPR1* using kanamycin (Kan, 100 mg l^{-1}, 172 μM) or A2C (250 μM) for selection. Shoots were regenerated from leaf explants in the presence of kanamycin or A2C, then transferred to selection rooting medium. (C) Brown root tips in one of the escape plantlets in A2C medium. The plates are 10 cm in diameter.

MPR1 *gene expression in the transgenic tobacco*

Northern blot analysis was performed on Southern-positive A2C-selected transformants to examine if the yeast-encoded *MPR1* gene could be successfully transcribed in tobacco. All 28 lines showed gene expression to various degrees, while the wild type did not show any detectable homologous *MPR1* transcript. A representative northern blot of 14 lines is shown in Fig. 5A. The size of the transcript falls between 1500 and 2000 nucleotides as estimated by the RNA size markers, larger than the expected 1033 nucleotides. A plausible explanation is that the transcription extended beyond the *NOS* terminator until the second *NOS* terminator in the *nptII* expression cassette located 678 bp downstream (Fig. 2), hence the longer transcripts. A similar phenomenon was observed in Zhang *et al.* (2004) where *MPR1* was under the control of the superpromoter (Ni *et al.*, 2005) and *NOS* terminator.

To confirm if the *MPR1* gene was translated into functional A2C acetyltransferase, leaf extracts from five transgenic lines exhibiting low to high gene expression levels in northern blot (Fig. 5A) were assayed for acetyltransferase activities using A2C and acetyl-CoA as substrates (Fig. 5B). The activities were measured as the rate of the subsequent reaction of the CoA-SH formed with DTNB, which could be monitored at 412 nm as the reaction product TNB formed. Other endogenous acteyltransferase activities were measured separately by excluding A2C from the reaction mixture and subtracted to obtain A2C-dependent acetyltransferase activities. No A2C-dependent enzyme activities were detected in the wild

Table 2. Transformation and selection efficiency of the *MPR1*/A2C selectable marker system in tobacco transformation

After transformation, shoots were regenerated from leaf explants and subsequently transferred to rooting medium under the selection pressure of 100 mg l^{-1} kanamycin or 250 μM and 300 μM A2C. Once rooted, plantlets were analysed by PCR for the presence of the *MPR1* transgene in their genome.

Selective agent	No. of rooted plantlets (A)	No. of PCR$^+$ plantlets (B)	No. of leaf explants (C)	Transformation frequency (B/C)	Selection efficiency (B/A)
Kanamycin 100 mg l^{-1}					
Rooted within 2 weeks	77	63			81.8%
2–3 weeks	15	5			33.3%
Total	92	68	14	485.7%	73.9%
A2C 250 μM					
Rooted within 2 weeks	183	52			28.4%
2–3 weeks	27	5			18.5%
3–4 weeks	60	6			10.0%
Total	270	63	294	21.4 %	23.3%
A2C 300 μM					
Rooted within 2 weeks	21	14			66.7%
2–3 weeks	18	7			38.9%
3–4 weeks	26	5			19.2%
Total	65	26	NA	NA	40.0%

NA, not available.

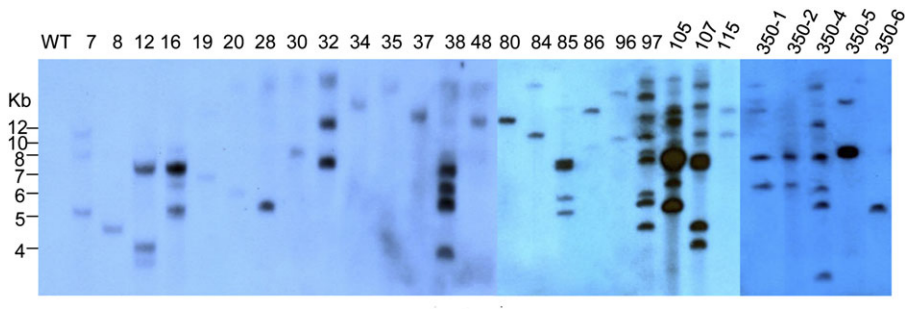

Fig. 4. Southern blot analyses of the *MPR1* transgenic tobacco obtained by A2C selection. Genomic DNA (20 μg) was digested with *Bam*HI and hybridized with an α-^{32}P-labelled *MPR1* cDNA probe after electrophoresis and blotting. Numbers on top represent individual transgenic lines. 350-1, 2, 4, 5, and 6 were obtained from 350 μM A2C selection, while the rest were selected by 300 μM. WT, wild type.

type, while the transgenic lines exhibited activity levels that correlated with the northern results (activities in 350-5 > 350-2 > #96 > #86 > #97). Together with the northern blot analysis, these results confirm that the introduced *MPR1* gene was successfully transcribed and translated into a functional enzyme that could carry out the acetylation reaction on A2C in *MPR1* transgenic tobacco plants.

Transgene segregation in the progeny monitored by A2C

Another important function of a selectable marker gene is to follow the transgene segregation in the progeny. To test if A2C selection could effectively identify the transgene-carrying progeny, the self-pollinated T_1 progeny seeds of four transgenic lines that showed single copy insertion in Southern hybridizations (Fig. 4) were grown on 400, 450, and 500 μM A2C or 250 mg l^{-1} (429 μM) kanamycin (Fig. 6) at a density of 24 seeds/plate. The same reduced inhibitory effect of A2C was observed as in the transformation process when the plates were overcrowded with seeds. Therefore, an equal number of seeds were placed on each plate with at least 1 cm space between each seed. The wild-type seeds were grown under the same treatments to serve as references representing the recessive progeny. As shown in Fig. 6, the wild type was not able to grow beyond the cotyledonary stage on kanamycin. A2C also had inhibitory effects on germination and growth; however, some seedlings were able to develop into complete seedlings at all three concentrations. Nevertheless, healthy seedlings could be easily distinguished from the smaller wild-type-like seedlings in the transgenic T_1 progeny (Fig. 6, lowest panel). The ratios of the healthy resistant to smaller sensitive seedlings were near 3:1 (Table 3), complying with Mendelian inheritance of a single gene.

To verify the accuracy of the visual identification, one plate was randomly chosen from each selection condition and all 24 seedlings were analysed by PCR with *MPR1*-specific primers. Except for one 400 μM-resistant seedling being PCR negative, all of the A2C-resistant seedlings were PCR positive. A few seedlings recorded as sensitive to A2C

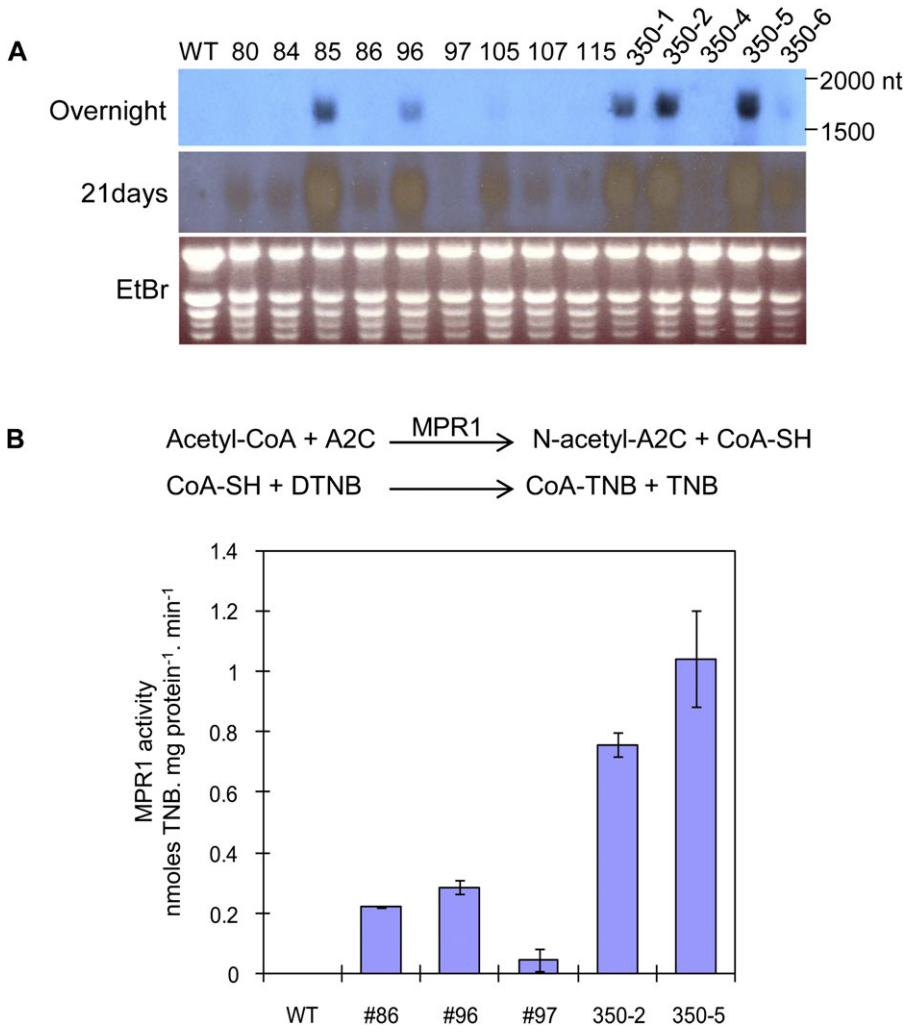

Fig. 5. *MPR1* gene expression in A2C-selected transgenic tobacco. (A) Northern blot analysis. Total RNA (40 μg) was electrophoresed on a 1.2% agarose–formaldehyde gel and probed with α-^{32}P-labelled *MPR1* cDNA. The X-ray film was developed after overnight (top) or 21 d (middle) exposure. The positions of the RNA size markers in nucleotides are indicated on the right. The ethidium bromide- (EtBr) stained gel before transfer is shown at the bottom. (B) Enzyme activity in the leaf extract was measured as the rate of A2C-dependent conversion of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). Results represent the average and standard deviation of three independent experiments. WT, wild type. Numbers represent individual *MPR1* transgenic lines.

were in fact PCR positive. Overall the error rates were 2/24, 1/24, and 2/24 at 400, 450, and 500 μM, respectively (Table 4). In comparison, kanamycin selection gave completely accurate results as all resistant seedlings were PCR positive and sensitive seedlings were PCR negative. Nevertheless, despite a few incorrectly recorded seedlings, A2C is highly effective in identifying transgene-carrying progeny.

Metabolite profiling of the MPR1 *transgenic plants*

Twenty-two independent *MPR1* tobacco lines and four wild-type plants were grown in a greenhouse until maturity. No phenotypic differences were observed in the transgenic plants when compared with the wild type. To investigate if *MPR1* gene expression has effects on plant metabolism, one young fully expanded leaf was collected from each 1-month-old plant and analysed by GC-MS metabolite profiling. Ninety-seven metabolites were identified (Table 5) and levels compared (data not shown). Unsupervised PCA of the data did not reveal any significant clusters (Fig. 7A), neither were clusters found when the wild type and *MPR1* were specified as two classes and subjected to partial least-squares discriminant analysis (PLSDA) (data not shown). When the highly variable metabolites serine, putrescine, pyruvate, and tyramine were omitted from the data set, unsupervised PCA did not show any clusters, but the wild type and *MPR1* formed two groups in supervised PLSDA (Fig. 7B) due to galactose levels. However, analysis of the galactose levels with *t*-test showed that the *MPR1* transgenic plants were not significantly different from the wild type ($p=0.066$, Fig. 7C). It was concluded that under normal growth conditions the expression of the *MPR1* gene in transgenic plants had no effect on metabolism.

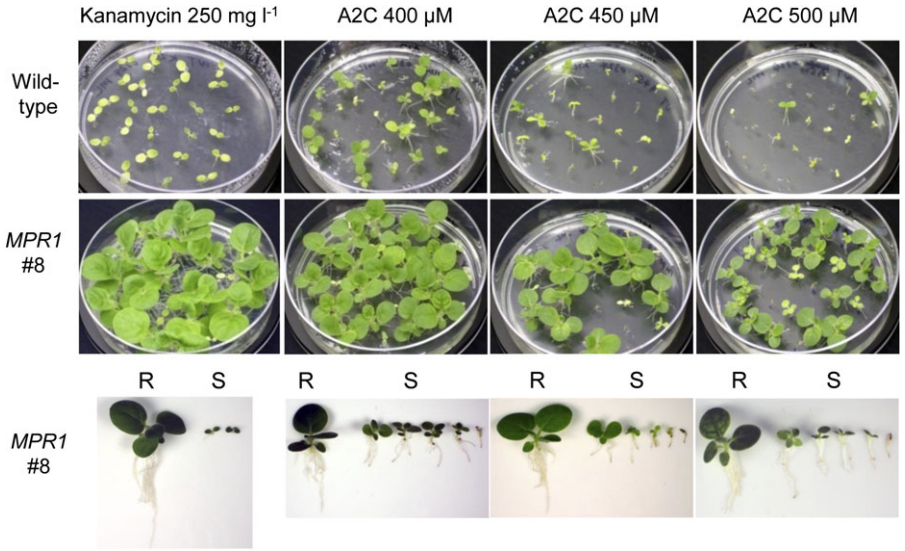

Fig. 6. Progeny segregation analysis. Seeds of the wild type or T_1 progeny of *MPR1* transgenic line #8 were grown on MS medium supplemented with A2C or kanamycin at the concentrations indicated at the top. The lowest panel shows the close-ups of the resistant (R) and sensitive (S) seedlings of *MPR1* #8 grown in selection conditions indicated at the top. Photographs were taken 21 d after sowing).

Table 3. Segregation analysis of the *MPR1* transgenic tobacco progeny

T_1 seeds were placed on medium supplemented with kanamycin (250 mg l^{-1}) or A2C (400, 450, and 500 μM) at a density of 24 seeds/plate. The numbers of resistant (R) and sensitive (S) seedlings were recorded after 2 weeks.

Line	Kanamycin 250 mg l^{-1}			A2C 400 μM			A2C 450 μM			A2C 500 μM		
	R	S	R:S	R	S	R:S	R	S	R:S	R	S	R:S
#8	165	66	2.5:1	123	42	2.93:1	74	22	3.36:1	117	51	2.29:1
#34	79	17	4.65:1	105	38	2.76:1	89	29	3.07:1	88	31	2.84:1
#80	98	44	2.23:1	126	42	3:1	122	46	2.65:1	124	44	2.82:1
350#6	147	44	3.34:1	76	23	3.3:1	116	52	2.23:1	111	56	1.98:1

The response of the MPR1 *transgenic tobacco under oxidative stress*

Studies in yeast provided evidence that *MPR1* imparts tolerance to oxidative stresses by reducing the levels of intracellular ROS (Nomura and Takagi, 2004; Du and Takagi, 2005, 2007). The proposed mechanism is that MPR1 is capable of acetylating and in turn reducing the levels of P5C, a toxic proline catabolism intermediate that has a similar structure to A2C (Fig. 1A). It has been reported that cellular accumulation of P5C could generate mitochondrial ROS (Miller *et al.*, 2009) and cause programmed cell death in plants (Deuschle *et al.*, 2004). In light of this evidence, experiments were conducted to investigate whether *MPR1* exerts the same protective effects in transgenic tobacco when under oxidative stress. For uniform application of the stress and easy measurement of the ROS levels and cell viability, suspension cell cultures were initiated from the wild type and five transgenic tobacco lines. When treated with H_2O_2, the viability of the wild type was not affected by up to 1 mM H_2O_2, although the ROS levels were elevated by ~20% compared with the untreated cells, suggesting that this treatment caused the formation of ROS (Fig. 8A). Two of the *MPR1* lines, 350-5 and #86, also remain vital at this concentration, while their ROS levels were significantly lower than those of the the wild type (Fig. 8A). In contrast, the viability of three other *MPR1* lines, 350-2, #96, and #97, was decreased by at least 40%. Although the ROS levels were lower in these lines, it was possibly due to a reduced number of viable cells in these lines and hence fewer ROS were generated. The inconsistent responses to H_2O_2 do not seem to correlate directly with the MPR1 enzyme activities, as two lines with similar activity levels (e.g. 350-2 and 350-5; #86 and #96, Fig. 8B) responded differently, and the high expresser line 350-2 showed a similar response to the lowest expresser #97. Overall, the transgenic lines showed responses different from those of the wild type, indicating that the *MPR1* transgene affected the response of the suspension cells to H_2O_2. Further investigation is necessary to understand the effects of MPR1 in plants in response to oxidative stress.

Discussion

MPR1 *and A2C can work as a selectable marker system for plant transformation*

In this study a selectable marker system for plant transformation was established using the yeast *MPR1* gene and the toxic proline analogue A2C. The results showed that *MPR1* was effectively expressed in the transgenic tobacco

Table 4. PCR analysis of the T_1 progeny segregating on kanamycin or A2C medium

Twenty-four of the *MPR1* #8 T_1 seeds were placed in each selection plate. Seedlings were recorded as resistant or sensitive before PCR analysis with *MPR1*-specific primers.

Selection conditions	Resistant	PCR$^+$	PCR$^-$	Sensitive	PCR$^+$	PCR$^-$	Error rate
A2C 400 μM	18	17	1	6	1	5	2/24
A2C 450 μM	17	17	0	7	1	6	1/24
A2C 500 μM	18	18	0	6	2	4	2/24
Kan 250 mg l^{-1}	18	18	0	6	0	6	0/24

Table 5. List of compounds measured by GC-MS in tobacco leaf extracts

O-Acetyl-L-serine	Aconitic acid	Alanine
Arabinose	Ascorbic acid	Aspartic acid
B-Alanine	1-Benzylglucopyranoside	Cadaverine
Caffeic acid	Chlorogenic acid	Cinnamic acid
Citric acid	Cytosine	*n*-Docosane
n-Dodecane	Erythritol	Ethanolamine
Ethyleneglycol	Fructose	Fumaric acid
GABA	Galactinol	Galactonic acid
Galactose	Galactose-6-P	Glucaric acid
Gluconic acid	Glucose	Glucose-6-P
Glutamic acid	Glyceric acid	Glycerol
Glycerol-3-P	Glycine	Glycolic acid
Glyoxilic acid	Gulonic acid	Hexonic acid
Hydroxylamine	4-Hydroxypyrydine	Inorganic phosphate
Inositol	Isoleucine	α-Ketoglutaric acid
α-Keto-L-gluconic acid	Ketomalonic acid	Lactic acid
Leucine	Linoleic acid	Linolenic acid
Maleic acid	Malic acid	Malonic acid
Mannitol	Mannose	2-Methylbenzoic acid
2-Methylmalic acid	myo-Inositol-2-P	Nicotine
n-Octacosane	*n*-Octadecane	*n*-Pentadecane
Octadecanol	3-PG	Phenylalanine
p-Hydroxybenzoic acid	Proline	Protocatechuic acid
Putrescine	Pyroglutamic acid	Pyruvic acid
Quinic acid	Rhamnose	Ribitol
Ribonic acid	Ribose	Sedoheptulose
Serine	Shikimic acid	Sorbitol
Sorbose	β-Sitosterol	Stigmasterol
Succinic acid	Sucrose	Tartaric acid
Threitol	Threonic acid	Threonic acid-1, 4-lactone
Threonine	Trehalose	Tyramine
Valine	Xylitol	Xylose
Xylulose		

and capable of carrying out an A2C-dependent acetylation reaction (Fig. 5), which in turn conferred resistance to A2C.

In a previous attempt to use this system for tobacco transformation, many untransformed cells were able to escape A2C selection, yielding a low selection efficiency of 4% (Zhang *et al.*, 2004). In this study the selection efficiency was improved to as high as 66.7% with 300 μM A2C (Table 2). The key for A2C to exert its best effect is to reduce the plant materials (14 leaf explants or seven regenerated shoots in one 10 cm plate) in the selection plates and subculture every 7–10 d during shoot regeneration. Studies in carrot tissue culture revealed that the growth-inhibitory effects of A2C can be reversed by increasing the concentration of proline in the culture medium (Widholm, 1976). It is possible that having too many explants or regenerated shoots on a plate leads to cross-feeding to increase the free proline levels and reverse the effect of A2C. In the rooting stage, shoots that rooted in the first 2 weeks had the highest PCR-positive rates (Table 2) so it is recommended to harvest shoots that root within 2 weeks. Plantlets with brown root tips (Fig. 3C) should be discarded as PCR analysis showed that these plantlets were escapes. The cause and nature of the brown root tips are unknown.

The results also showed that transgene segregation in the T_1 progeny can be monitored by 400, 450, and 500 μM A2C (Fig. 6). A2C did not completely inhibit the growth of the recessive progeny as did kanamycin, but it was inhibitory enough to allow the dominant and recessive progeny to be distinguished. For the best results, seeds should be kept at least 1 cm apart in the selection plates. In the present experiments, 24 seeds were placed in a 10 cm Petri dish and the results were satisfactory (Table 4).

When grown in a greenhouse, the growth and phenotypes of 22 independent *MPR1* transgenic lines appeared normal. Further analysis of these plants with GC-MS metabolic profiling did not detect any significant changes in the 97 metabolites analysed when compared with the wild type (Fig. 7). This suggests that *MPR1* expression does not alter the metabolism or growth and development of transgenic plants under normal conditions.

Although the *MPR1*/A2C selection system is not as efficient as *nptII*/kanamycin (Table 2, Table 4), it has the advantage of not involving the use of a microbial antibiotic resistance gene, and therefore not posing a threat to the environment, and should not be objectionable to the public. The next step is to determine if this system can be applied in economically important crops such as soybean and corn.

Possible function of MPR1 in preventing the accumulation of the proline catabolite P5C

Recent studies in yeast suggest that the proline biosynthesis/catabolism intermediate, P5C, or more probably its spontaneous equilibrium form GSA, is the natural substrate for MPR1 in yeast (Fig. 1A, Nomura and Takagi, 2004). Proline biosynthesis is a two-step reaction that occurs in the

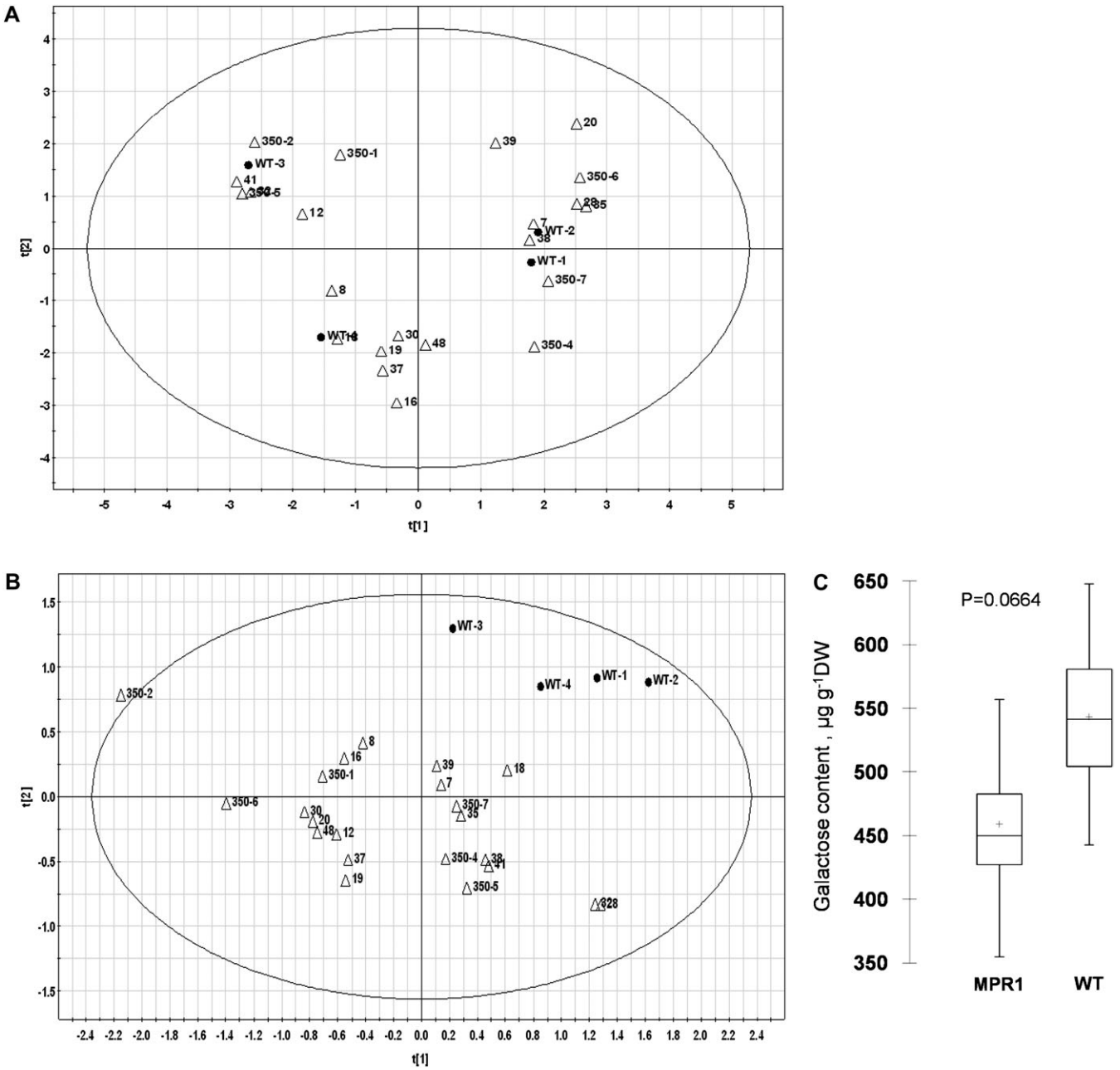

Fig. 7. (A) Unsupervised PCA of the GC-MS profiling data. (B) Supervised PLSDA analysis with data excluding the highly variable metabolites serine, putrescine, pyruvate, and tyramine. (C) *t*-test analysis of the galactose levels. WT (filled circles), wild type. Numbers indicate individual *MPR1* transgenic lines (open triangles).

cytosol (and also in plastids in plants). The starting substrate glutamate is first converted to P5C by P5C synthetase (P5CS) then subsequently reduced to proline by P5C reductase (P5CR). Proline catabolism is the reverse process that takes place in the mitochondria, where proline is oxidized to P5C and then to glutamate by proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH), respectively (reviewed in Verbruggen and Hermans, 2008). It has been reported that excess accumulation of P5C during proline oxidation can lead to the generation of ROS and result in cell death in human cells (Donald *et al.*, 2001) and plants (Deuschle *et al.*, 2004; Miller *et al.*, 2009). Nomura and Takagi (2004) suggested that MPR1 can acetylate excess P5C/GSA and consequently prevent ROS production and the resultant cell death in yeast. However, MPR1 lacks the mitochondria-targeting sequence and appears to be a cytosolic enzyme, thus it should not have access to the mitochondrial P5C/GSA. The authors proposed that P5C is either leaked to the cytosol due to the change of mitochondrial permeability caused by P5C/GSA accumulation, or transported from the mitochondria to the cytosol so that MPR1 can acetylate P5C in the cytosol (Nomura and Takagi, 2004).

Transport of P5C from the mitochondria to the cytosol is likely in plants. First of all plants are known to have an alternative proline biosynthesis route, which uses ornithine as

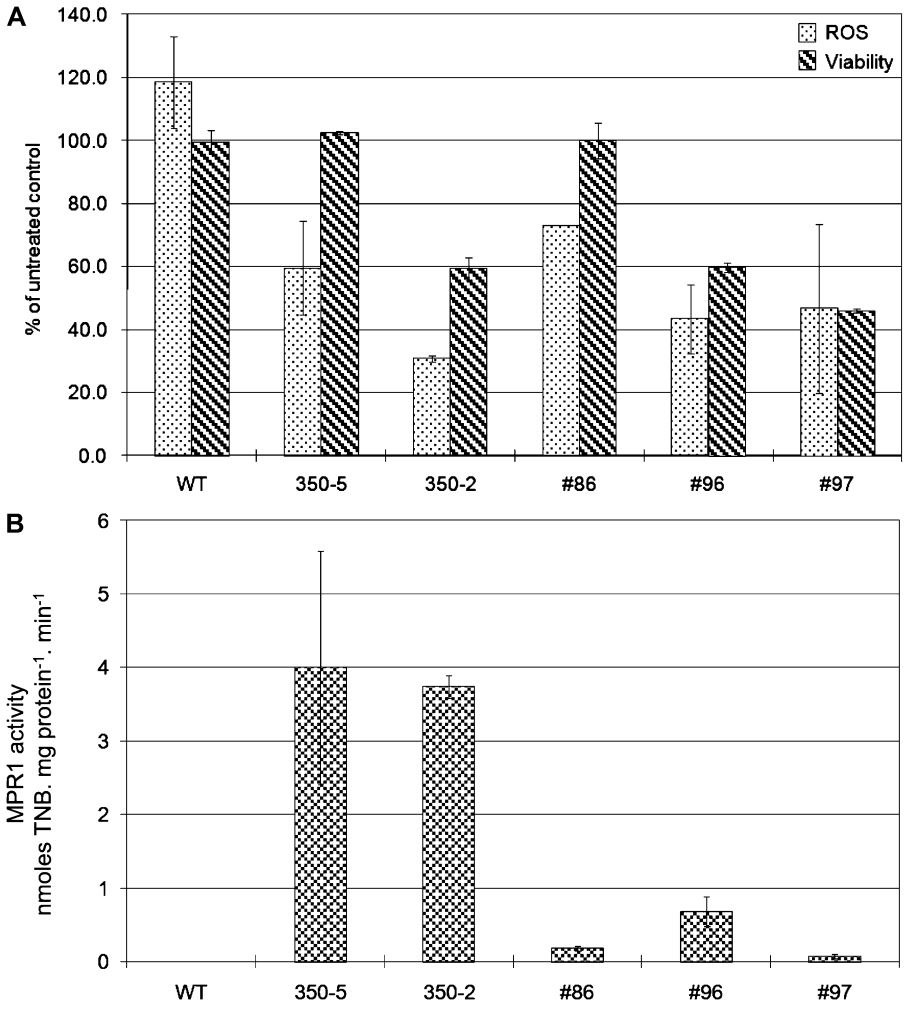

Fig. 8. (A) Effects of H_2O_2 treatment on the levels of reactive oxygen species (ROS) and viability of *MPR1* transgenic tobacco cells. After 45 min treatment with 1 mM H_2O_2, intracellular ROS levels were measured by quantifying 2′,7′-dichlorofluorescein fluorescence. Cell viability was determined spectrophotometrically using an MTS tetrazolium-based assay. The results are expressed as a percentage of the untreated controls. Data represent the average and standard deviation of two independent experiments conducted in triplicate. (B) MPR1 enzyme activities in the *MPR1* transgenic suspension cells. Enzyme activities were measured as the A2C-dependent formation of TNB from DTNB, as described in Fig. 5B. Data represent the average and standard deviation of three independent measurements. WT, wild type. Numbers at the bottom represent individual transgenic lines.

a precursor to synthesize P5C by ornithine-δ-aminotransferase in the mitochondria. P5C is then reduced to proline in the cytosol or chloroplasts (Verbruggen and Hermans, 2008). Furthermore, a recent study in tobacco and *Arabidopsis* suggested a P5C–proline cycle operating between the mitochondria and cytosol that functions to maintain the cellular P5C/proline ratio (Miller *et al.*, 2009). The authors demonstrated that the P5C and proline ratios remained constant under stress conditions in transgenic tobacco that overexpresses ProDH and in the *Arabidopsis p5cdh* mutant that is impaired in P5CDH, when P5C was expected to accumulate in both cases. It is proposed that during the degradation of stress-accumulated proline, when excess P5C is generated and not rapidly oxidized to glutamate in the mitochondria, P5C is recycled back to the cytosol where it is reduced to proline, thus preventing the build-up of P5C in the mitochondria. Proline subsequently returns to the mitochondria for degradation. The proline oxidation reaction carried out by ProDH transfers two electrons to the mitochondrial electron transport chain then to O_2, resulting the formation of ROS. In summary, as a consequence of excess accumulation of proline in response to stress, the P5C–proline cycle operates intensively and releases electrons that lead to the generation of ROS, and triggers programmed cell death (Miller *et al.*, 2009). A similar proline cycle has also been proposed in human cells (Yoon *et al.*, 2004). To prove further the existence of such cycle, the transporter responsible for shuttling P5C needs to be identified.

The effect of MPR1 *expression in transgenic plants under stress conditions remains to be assessed*

To investigate if MPR1 can exert protective effects against oxidative stress in the transgenic tobacco as in yeast, stress was produced by H_2O_2 treatment of suspension culture cells of five *MPR1* transgenic lines. Two lines showed lower ROS

levels than the wild type, supporting the postulated protective effect of MPR1, whereas the other three showed decreased viability but did have lower ROS levels (Fig. 8A). The somewhat contradictory results were not directly correlated to the MPR1 enzyme activity levels, suggesting that other factors are possibly involved. This is not surprising as plants possess more complex signalling pathways than yeast in order to cope with various stress conditions. In addition, the results obtained from suspension cultures may not reflect the response of a whole plant. Further experiments examining the responses of whole *MPR1* transgenic plants against certain stresses that crop plants normally encounter in the growth environment are necessary.

Acknowledgements

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