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ORIGINAL RESEARCH PAPER

Overexpression of an HPS/PHI fusion enzyme from *Mycobacterium gastri* in chloroplasts of geranium enhances its ability to assimilate and phytoremediate formaldehyde

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Abstract 3-Hexulose-6-phosphate synthase (HPS) and 6-phosphate-3-hexuloisomerase (PHI) are two key enzymes in the formaldehyde (HCHO) assimilation pathway in methylotrophs. The HPS/PHI fusion protein, encoded by the chimeric gene of *hps* and *phi* from *Mycobacterium gastri* MB19, possesses both HPS and PHI activities in an *Escherichia coli* transformant. Overexpression of the fusion protein in chloroplasts of geranium (*Pelargonium* sp. Frensham) created a photosynthetic HCHO assimilation pathway according to ^{13}C-NMR analysis. The transgenic plants exhibited an enhanced ability in HCHO-uptake and [^{14}C]HCHO-assimilation. Moreover, the transgenic plants showed greater HCHO-resistance and stronger capacity in purification of the HCHO-polluted air. Therefore, the use of the single chimeric gene may not only greatly simplify the transformation procedure but also improve the efficiency of phytoremediating HCHO in ornamental plants.

Keywords Formaldehyde assimilation pathway · Geranium · Photosynthesis · Phytoremediation · Transgenic plant

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Introduction

Formaldehyde (HCHO) is a major indoor air pollutant. It exists in vivo at low levels in organisms because it is a key metabolic intermediate of one-carbon (C1) metabolism. Plants can remove HCHO from contaminated air (Wolverton et al. 1984) and exogenous HCHO can be incorporated into the C1 metabolism of photosynthetic cells. For example, feeding the common spider plant (*Chlorophytum comosum*) with [^{14}C]HCHO resulted in ^{14}C-labeled products derived from C1 metabolism (Giese et al. 1994). Furthermore, genetic engineering has been used to enhance the limited capacity of plants to remove HCHO. Overexpression of a glutathione-dependent HCHO dehydrogenase (FALDH) in *Arabidopsis* caused a significant increase in HCHO-absorption (Achkor et al. 2003).

The ribulose monophosphate pathway (RuMP) is a highly developed mechanism to detoxify HCHO in

methylotrophic bacteria. In this pathway, HCHO is fixed to D-ribulose 5-phosphate (Ru5P) to form D-arabino-3-hexulose 6-phosphate, which is then isomerized to fructose-6-phosphate (F6P). These two sequential reactions are catalyzed by 3-hexulose-6-phosphate synthase (HPS) and 6-phosphate-3-hexuloisomerase (PHI), respectively (Kato et al. 2006). A chimeric gene (*rmpAB*) of *hps* and *phi* from *Mycobacterium gastri* MB19 was successfully expressed in *Escherichia coli* and the expressed fusion protein HPS/PHI was fully active at room temperature. The activity of the fusion enzyme was significantly greater than a mixture of the two separate enzymes. The *E. coli* transformants assimilated HCHO efficiently and exhibited better growth in HCHO-containing medium than the host strain (Orita et al. 2007).

Since the substrate (Ru5P) and product (F6P) of the sequential reactions catalyzed by HPS and PHI are intermediates of the Calvin cycle in plants, photosynthesis could provide sufficient substrates for the reactions catalyzed by HPS and PHI if the two enzymes were expressed in plant chloroplasts. Our recent study had revealed that overexpression of the HPS and PHI from *M. gastri* MB19 in transgenic *Arabidopsis* and tobacco chloroplasts established a photosynthetic HCHO assimilation pathway, and thereby enhanced the ability of the plants to absorb and assimilate exogenous HCHO (Chen et al. 2010). Transformants with both HPS and PHI activity can be obtained by introducing the *rmpAB* chimeric gene in a one-step procedure. Therefore, the *rmpAB* gene is an attractive candidate for genetic engineering to install a photosynthetic HCHO assimilation pathway (Fig. 1a) in ornamental plants for phytoremediation of HCHO pollution. Here, we generated transgenic geranium (*Pelargonium* sp. frensham) plants overexpressing the HPS/PHI fusion protein in the chloroplasts to test our hypothesis.

Materials and methods

Vector construction

The *rmpAB* gene was amplified by PCR using the plasmid pEThps-phi (Orita et al. 2007) as the template with the upstream primer rmpA-F (5′-CACC GCATGCAGCTCCAAGTCGCCATCG-3′, *Sph*I site underlined) and the downstream primer rmpB-R,

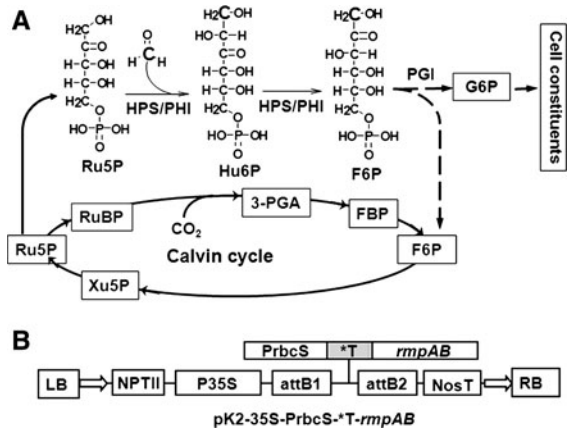

Fig. 1 Diagram of strategy (**a**) and DNA construct (**b**) for installation of photosynthetic HCHO assimilation pathway (HPS and PHI) into chloroplasts of geranium plants. *Ru5P*, D-ribulose 5-phosphate; *Hu6P*, D-arabino-3-hexulose 6-phosphate; *F6P*, fructose 6-phosphate; *Xu5P*, xylulose 5-phosphate; *RuBP*, ribulose 1,5-bisphosphate; *3-PGA*, glycerate 3-phosphate; *FBP*, fructose-1,6-bisphosphatase; *PGI*, phosphoglucose isomerase; *PrbcS*, tomato rbcS-3C promoter; **T*, tomato rbcS-3C transit peptide sequence with *Nco*I site in ATG start codon; *rmpAB*, chimeric gene encoding HPS/PHI; LB and RB, left border and right border; *NPTII*, Kan-resistance gene; *P35S*, CaMV 35S promoter; *attB1 and attB2*, two specific attachment sites for Gateway BP reaction; *NosT*, nopaline synthase terminator

(5′-TCTAGATCACTCGAGGTTGGCGTGGCGCG-3′, *Xba*I site underlined). The PCR product was cloned into pMD18-T (TaKaRa) to generate pMD18-*rmpAB*. The sequence of the *rmpAB* gene was verified by DNA sequencing. A modified Gateway entry vector pENTR*-PrbcS-*T-GFP was used that contains a light-inducible promoter (PrbcS) of Rubisco small subunit 3C and its transit peptide sequence (*T) from tomato (Sugita et al. 1987). The *rmpAB* DNA fragment was excised from pMD18-*rmpAB* with *Sph*I and *Eco*RI and inserted into the same restriction sites of pENTR*-PrbcS-*T-GFP to yield the pENTR*-PrbcS-*T-*rmpAB* entry clone. The plant expression vector pK2-35S-PrbcS-*T-*rmpAB* was derived by subcloning the PrbcS-*T-*rmpAB* segment from pENTR*-PrbcS-*T-*rmpAB* into the pK2GW7 destination vector (Karimi et al. 2002) with a Gateway LR Clonase II Enzyme Mix kit (Invitrogen) according to the manufacturer's instructions.

Transformation of geranium

The plant expression vector pK2-35S-PrbcS-*T-*rmpAB* was transferred into *Agrobacterium tumefaciens* C58C1

(pMP90) following the procedures by Chen et al. (2010). Transformation of geranium (*Pelargonium* sp. Frensham) was performed following the leaf petiole co-cultivation protocol (KrishnaRaj et al. 1997). Resistance of transformants to antibiotics was determined by testing their growth on MS medium (1% sucrose) containing 50 μg kanamycin/ml.

Genomic PCR and western blot analysis

Genomic DNA was prepared from plant leaves as described elsewhere (Murray and Thompson 1980). Using genomic DNA as the template, PCR analysis was conducted with rmpA-F and rmpB-R primers to detect integration of the *rmpAB* gene.

Protein samples were prepared from fresh leaf tissues using a Plant Total Protein Extraction kit (Sigma). For Western blot analysis, 15 μg total proteins was separated by SDS-PAGE (12%) and then transferred to PVDF-P membranes. The membranes were first treated with rabbit antibodies against *Mycobacterium gastri* MB19 HPS or PHI (Orita et al. 2007), and then with a goat antibody against rabbit IgG conjugated with peroxidase.

HCHO-tolerance, HCHO-uptake and HCHO assimilation analysis

All investigations were under the constant light (100 μmol m^{-2} s^{-1}) at 25°C. The detailed procedure was performed as described by Chen et al. (2010).

Analysis of gaseous HCHO uptake

To measure the uptake rate of gaseous HCHO, the experiment was carried out in a specific, 294-l glass chamber (700×600×700 mm; see Supplementary Fig. 1a). The light (75 μmol m^{-2} s^{-1}) was supplied from lamps installed on two sides of the chamber. Four small fans were installed in the corners of the chamber, to circulate the gaseous HCHO. The temperature and humidity in the chamber were monitored by a sensor and displayed automatically (Supplementary Fig. 1a). The plant pots were covered with plastic bags (Supplementary Fig. 1b) and then placed into the chamber via a door at the front of the chamber. Air-polluting HCHO was then introduced into the chamber by a pump, or injected into the chamber using a syringe. The gaseous HCHO concentration in the chamber was detected using a gaseous HCHO sensor (CH2O/C-10, Membrapor, Swizerland). The sensor was connected to a monitor, the sensor outputs were held by a data logger, and the data were processed using a computer. The measurements were initiated when the HCHO concentration was close to 5 ppm. To maintain HCHO in a gaseous state, the temperature and the humidity of the chamber were controlled at 26–32°C and 25–60%, respectively, over the whole measurement period.

After measurements, all leaves were collected from the plants and scanned together with a piece of paper on a scanner to determine the total leaf area for the plants on each pot. The scanned picture was used to obtain the pixels of leaves (m) and the paper (n) using software, respectively. The square of the paper was measured with a ruler and defined as Ap. The leaf area (Al) was calculated by the formula $Al = m/n \times Ap$.

[^{13}C]HCHO and [^{13}C]$NaHCO_3$ labeling experiments

For [^{13}C]HCHO labeling experiments, aseptic geranium leaves (2 g) were soaked in 70 ml of 2 mM $H^{13}CHO$ solution (containing 5 mM $KHCO_3$ and 0.1% MES (w/v), pH 5.7). For $NaH^{13}CO_3$ labeling experiments, geranium leaves (2 g) were harvested from plants grown in soil in pots. After surface disinfection (Itoh et al. 1998), the leaves were soaked in 70 ml 5 mM [^{13}C]$NaH^{13}CO_3$ solution (containing 0.1% MES, pH 5.7) with or without 2 mM HCHO. The labeling experiments were performed under constant light (100 μmol m^{-2} s^{-1}) with shaking (100 rpm) at 25°C. The sample was prepared following the previous method (Chen et al. 2010).

NMR analysis

^{13}C NMR data was collected using described parameters (Chen et al. 2010). For samples with [^{13}C]HCHO labeling, chemical shifts were obtained by reference to formamide resonance at 166.85 ppm. For samples with [^{13}C]$NaHCO_3$ labeling, chemical shifts were obtained by reference to maleic acid at 130.66 ppm. The resonances were assigned based on previously published results (Yanase et al. 1992; Yasueda et al. 1999) and confirmed by the chemical shifts of authentic samples. For calculation of the relative content of [1-^{13}C]F6P and other metabolites, the target

peaks were integrated relative to formamide as an external reference. The formamide was sealed in a capillary that was inserted into the 5-mm tube containing the sample.

Results and discussion

Generation of transgenic *rmpAB* geranium expressing HPS/PHI fusion protein

The DNA construct for *rmpAB* is shown in Fig. 1b. Using genomic PCR analysis, the presence of the *rmpAB* in the genome of transgenic geranium was confirmed in ten of the 15 kanamycin-resistant lines (Fig. 2a). Total protein was extracted for Western analysis to investigate the expression of HPS/PHI protein. As shown in Fig. 2b, a ~45 kDa band was detected in the protein samples from the three selected transgenic lines and the positive control but not in that from WT plants. The molecular mass of the HPS/PHI protein, which was identical to that of the protein expressed in *E. coli,* confirmed the correct removal of the transit peptide. This implied that the expressed HPS/PHI protein in the transgenic plants was delivered into the stroma of chloroplasts. These results demonstrated that the HPS/PHI fusion protein was successfully expressed in the leaves of the transgenic lines.

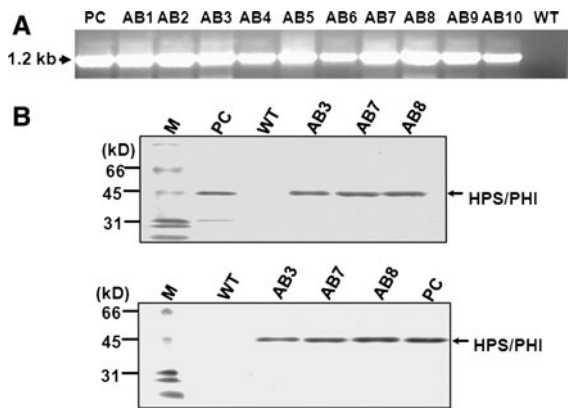

Fig. 2 Detection of *rmpAB* integration (**a**) and HPS/PHI protein expression (**b**) in transgenic geranium plants. For PCR analysis, positive control was performed using pK2-35S-PrbcS-*T-*rmpAB* as template. For Western blot analysis, HPS antibody (*upper panel*) and PHI antibody (*lower panel*) was used to detect expression of HPS/PHI fusion protein. Crude protein (5 μg) prepared from *E. coli* cells expressing HPS/PHI was used as positive control. *AB1-AB10*, transgenic geranium lines; *WT*, wild type geranium plants; *PC*, positive control; *M*, protein markers (biotinylated)

[^{13}C]HCHO metabolic profiling in transgenic geranium

Using ^{13}C-NMR, previous studies revealed that the mixture of HPS and PHI catalyzed the reaction of [^{13}C]HCHO and Ru5P to generate [1-^{13}C]F6P in vitro (Yanase et al. 1992; Yasueda et al. 1999). This compound is deduced as the initial [^{13}C]HCHO assimilation product of the engineered HPS/PHI pathway. As shown in Fig. 3, a strong resonance signal peak corresponding to [1-^{13}C]F6P with a chemical shift at 62.58 ppm was observed in the transgenic line spectrum, whereas only a weak signal was detected in the spectrum of the WT extract. The relative content of [1-^{13}C]F6P increase 150% in transgenic plants compared to the WT plants. This result indicates that the expressed HPS/PHI protein is active and more HCHO is fixed onto Ru5P to produce F6P in transgenic geranium. The HCHO assimilation pathway was successfully introduced into the transgenic plants.

Several other resonance peaks (U3–U11 in Fig. 3a) were also enhanced in transgenic sample. These resonances correspond to other phosphate sugars in the Calvin cycle and showed 30–180% increases compared with the WT sample. In addition, a strong resonance signal peak corresponding to α-D-[1-^{13}C] glucose 6-phosphate (G6P) with chemical shift at 92.13 ppm and a weak resonance signal peak with chemical shift at 95.67 ppm corresponding to *β*-D-[1-^{13}C]G6P appeared in the spectrum of the transgenic extract (Fig. 3a). These two peaks were very weak or undetectable in the WT sample (Fig. 3b). These data indicated that the initial [^{13}C]HCHO assimilation product, [1-^{13}C]F6P, was subsequently synthesized into [1-^{13}C]G6P and other phosphate sugars in the Calvin cycle. Based on the evidence, it appears that some of the [1-^{13}C]F6P is recycled in the Calvin cycle for generation of Ru5P. This verifies that the engineered HPS/PHI pathway is a photosynthetic HCHO assimilation pathway.

Two strong resonance signals, U1 and U2, with chemical shifts at 56.77 and 17.06 ppm, respectively, were observed in the ^{13}C-NMR spectrum of the WT sample (Fig. 3b), but were absent from that of the

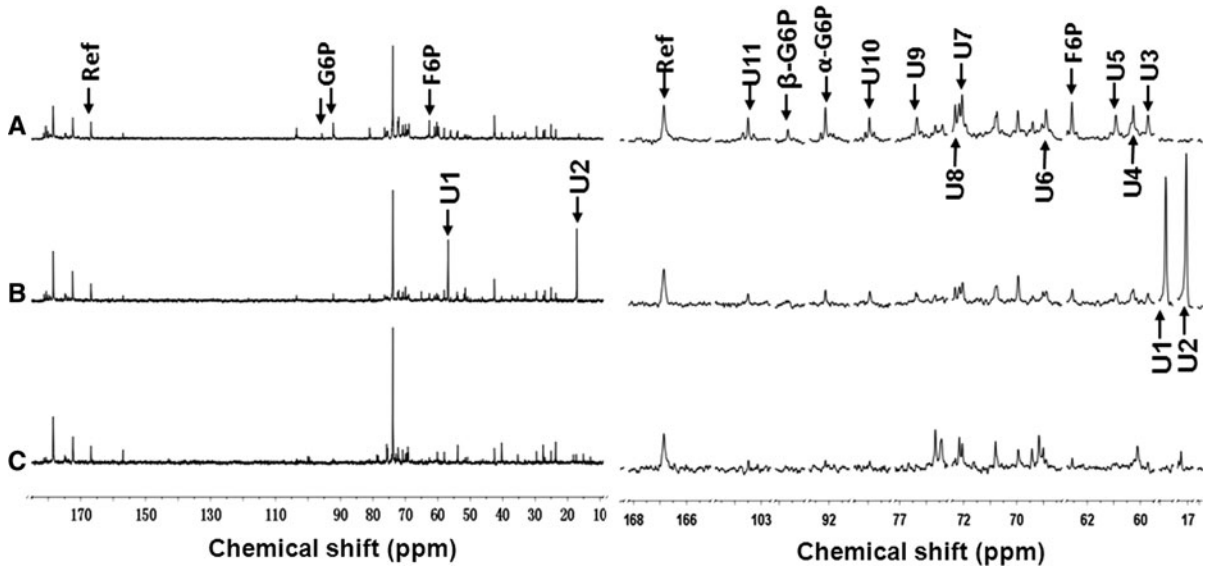

Fig. 3 Analysis of [^{13}C]HCHO metabolite profiling in transgenic and WT geranium plants with ^{13}C-NMR. Complete ^{13}C-NMR spectra (*left*) and expanded regions of interest (*right*) of leaf extracts of AB7 (**a**) and WT (**b**) treated with H^{13}CHO for 24 h. The extract from WT plant leaves without [^{13}C]HCHO treatment was used to monitor the background ^{13}C-NMR signal levels of geranium leaves (**c**). Peak assignments are as follows: Ref, formamide; *β*-G6P, *β*-D-[1-^{13}C]G6P; α-G6P, α-D-[1-^{13}C]G6P; F6P, [1-^{13}C]F6P; U1–U11, unknown signals

AB7 sample (Fig. 3a). We have not yet determined the identity of these resonance signals in our experiments. However, it is clear that these signals represent major metabolites of [^{13}C]HCHO metabolism in WT geranium. This suggests that the metabolism of [^{13}C]HCHO by the installed HPS/PHI pathway is efficient, directing most of the [^{13}C]HCHO flux towards itself. Furthermore, the photosynthetic CO_2 assimilation was not affected in transgenic plants according to the [^{13}C]$NaHCO_3$ metabolic profiling in the presence or absence of HCHO (Supplementary Fig. 2).

Augmentation of purification capacity for HCHO-polluted air in the transgenic geranium

The uptake rate of the transgenic plants for gaseous HCHO in the polluted air is an important indicator of their phytoremediation efficiency. As shown in Fig. 4a, the time required for WT, AB3, AB7 and AB8 plants to consume 50% of the initial HCHO was 32, 14, 5 and 5 min, respectively. To remove all of the gaseous HCHO, the time required for AB3, AB7 and AB8 plants was 50, 30, and 55 min, respectively. However, 0.7 ppm HCHO remained after WT plants had been in the chamber for 55 min. The uptake rate of gaseous HCHO for WT, AB3, AB7 and AB8 plant leaf was 1.64, 2.71, 3.41 and 2.04 ppm m^{-2} min^{-1}, respectively. These results indicated that the purification capability of the transgenic plants was higher than that of WT plants. Thus, installation of the HPS/PHI pathway into geranium plants increased their ability to remove gaseous HCHO from the atmosphere.

Enhancement of aqueous HCHO-uptake and assimilation in transgenic geranium

We also investigated the rate of transgenic plants to absorb aqueous HCHO in HCHO solution (Fig. 4b). The transgenic plant leaves showed a higher uptake rate than the WT plant leaves. After 90 h, the three tested transgenic lines completely removed HCHO from the solution, while 17% of the total HCHO remained in the WT sample. The control with no leaves showed no change in HCHO levels over the course of the experiment (data not shown).

[^{14}C]HCHO tracer experiment was used to investigate the HCHO assimilation ability of the plants. As shown in Fig. 4c, the radioactivity of the TCA-insoluble fraction from the transgenic plants was approximately 30% higher than that from WT plants. This result confirmed that overexpression of the

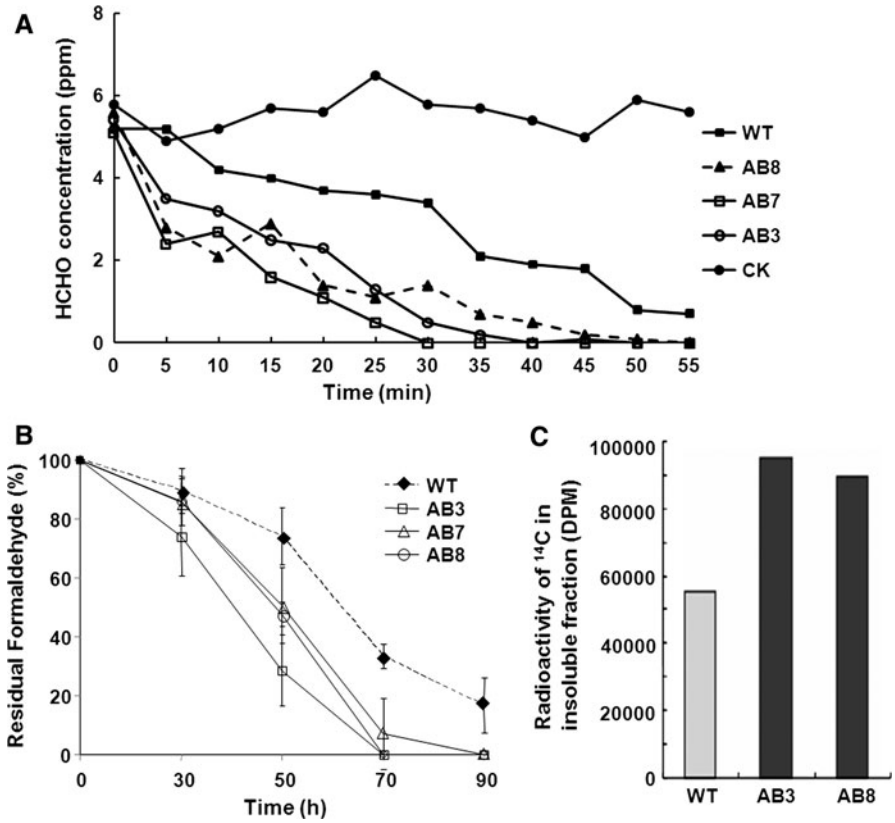

Fig. 4 Elimination and assimilation of HCHO from environment by transgenic geranium plants. **a** Kinetics of gaseous HCHO elimination by transgenic geranium plants. Geranium plants were grown in pots in a greenhouse for 2–3 months, and plants of equivalent size were used for analyses. Experiment was repeated more than three times and representative results are shown. CK, a soil-containing pot without plants (Supplementary Fig. 1b). **b** Uptake of aqueous HCHO by transgenic lines. Geranium leaves (2 g) was soaked in 150 ml HCHO solution (containing 4 mM HCHO, 5 mM $KHCO_3$ and 0.1% MES (w/v)) in a flask. Data are shown as percentage HCHO remaining in solution at indicated times during experiment. **c** Assimilation of aqueous HCHO by transgenic plants. Geranium leaves (0.5 g) were submerged in solution, 10 ml in total, containing 5 mM $KHCO_3$, 2 mM HCHO, 0.1% MES (w/v, pH 5.7) and 2.0 μCi [^{14}C]HCHO. After treatment for 24 h, the insoluble fraction was obtained by trichloroacetic acid extraction, and its radioactivity was measured. Experiment was repeated three times and representative results are shown

fusion HPS/PHI protein allowed transgenic geranium plants to assimilate exogenous HCHO into cell constituents more efficiently than WT plants. This probably resulted from the incorporation of the F6P produced from the engineered HPS/PHI pathway into cell constituents. The studies conducted by Jones et al. (1986) and Yu et al. (2000) showed that a decrease in the activity of chloroplast phosphoglucose isomerase (PGI), which catalyzes the reversible isomerization of F6P and G6P, affected the synthesis of leaf starch. Accordingly, we deduced that F6P derived from the engineered photosynthetic HCHO assimilation pathway was possibly converted to G6P by chloroplast PGI. As demonstrated by Alonso et al. (2005), G6P is subsequently used to synthesize starch and cell wall constituents. This may explain the accumulation of radioactivity in the insoluble fraction of the transgenic plants.

Improvement of HCHO-tolerance in transgenic geranium

The tolerance of plants to aqueous HCHO was tested on MS medium containing 7 mM HCHO. After growth on this medium for 15 days, most of the WT leaves became yellow and necrotic while the transgenic leaves remained green (Fig. 5a). Similarly, under gaseous HCHO treatment, the transgenic line

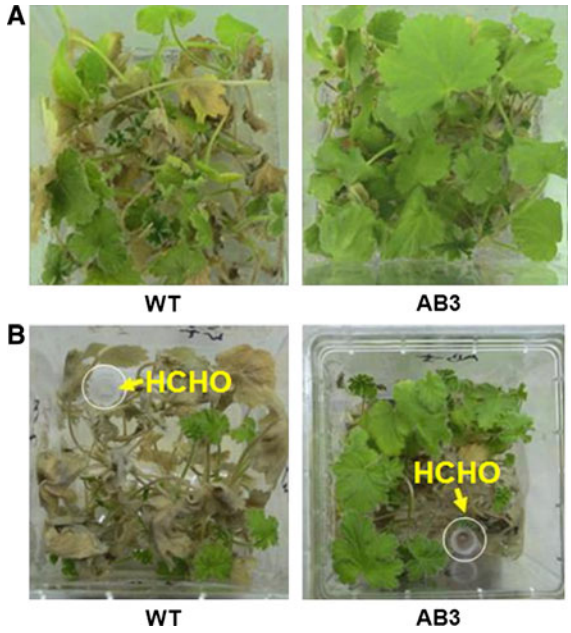

Fig. 5 Tolerance of transgenic geranium plants to aqueous (**a**) and gaseous (**b**) HCHO. To test tolerance to aqueous HCHO, equal numbers of geranium shoots were cut and transferred to MS medium containing 7 mM HCHO. For gaseous HCHO tolerance analysis, geranium shoots were transferred into boxes (370 ml) and grown for 3–4 weeks. A microcentrifuge tube (500 µl) containing 30 µl HCHO (37%, w/w) was then placed in the box. These results were photographed after 2 weeks of treatment

showed better growth than the WT plants. Almost all of the WT leaves withered while most of the transgenic plant leaves remained green and appeared healthy (Fig. 5b). These results indicated overexpression of the HPS/PHI improved the HCHO-tolerance in transgenic plants. It was reported that plants exposed to high concentrations of exogenous HCHO showed damage to chloroplasts and consequently, leaf bleaching symptoms (Achkor et al. 2003). The rapid fixation of toxic HCHO by the HPS/PHI pathway in chloroplasts might prevent the destruction of chloroplasts caused by HCHO, and thus enhance the HCHO-tolerance of transgenic geranium. HCHO is toxic to plants even at low concentration (Mutters et al. 1993). The stomatal conductance was decreased by 50% in *Ficus benjamina* when exposed to 0.05 ppm HCHO of realistic polluted indoor conditions for several weeks (Schmitz et al. 2000). The rate of HCHO uptake through the stomata is considered as a major limiting factor for phytoremediation of HCHO (Schmitz et al. 2000). Stomatal cells also contain chloroplasts and thus the installed photosynthetic HCHO assimilation pathway functions in stomatal cells. The concentration of HCHO in stomatal cells can be kept at very lower level when transgenic plants are used in phytoremediation of HCHO. This would weaken the impairment of HCHO to stomatal conductance in transgenic geranium leaves. As a result, transgenic plants may function in phytoremediation for a longer time than WT plants in indoor environments of low-level HCHO.

Conclusion

Geranium is a garden plant that is easy to cultivate and is grown worldwide. Thousands of Geranium cultivars have been developed. The cultivar *Pelargonium* sp. 'Frensham' is an easily transformed variety, and was used as the transformation material in the present study because it does not produce seeds and can be propagated easily through tissue culture (KrishnaRaj et al. 1997; Dan et al. 2000). Since there is no risk of ecological problems caused by its pollen, it would have wider acceptability to the public as a functional ornamental to remediate HCHO pollution. The HCHO-purification capacity of transgenic geranium is stronger than that of the WT. This demostrates that installation of the photosynthetic HCHO assimilation pathway in plants is an effective strategy for remediation of HCHO pollution. Therefore, the use of *rmpAB* fusion gene provides a quick and convenient approach for molecular breeding to improve the HCHO phytoremediation of ornamental plants. Furthermore, methanotrophic bacteria assimilate methane by converting it to HCHO via methane monooxygenase and methanol dehydrogenase (Hanson and Hanson 1996). This implies that further expression of these enzymes in our transgenic plants may yield plants that can assimilate methane. This is one approach that could be used to prevent increases in this greenhouse gas in the Earth's atmosphere.

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