Assimilation of Formaldehyde in Transgenic Plants Due to the Introduction of the Bacterial Ribulose Monophosphate Pathway Genes

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Formaldehyde (HCHO) is an air pollutant suspected of being carcinogenic and a cause of sick-house syndrome. Microorganisms called methylo trophs, which can utilize reduced C1 compounds such as methane and methanol, fix and assimilate HCHO, whereas most plants are unable to assimilate HCHO directly. We found that a bacterial formaldehyde-fixing pathway (ribulose monophosphate pathway) can be integrated as a bypass to the Calvin-Benson cycle in transgenic Arabidopsis thaliana and tobacco by genetic engineering. These plants showed enhanced tolerance to HCHO and enhanced capacity to eliminate gaseous HCHO by fixing it as a sugar phosphate. Our results provide a novel strategy for phytoremediation of HCHO pollution, and also represent the first step toward the production of plants that can assimilate natural gas-derived C1 compounds.

Key words: Arabidopsis thaliana; Calvin-Benson cycle; formaldehyde; ribulose monophosphate pathway; transgenic plant

Formaldehyde (HCHO), industrially produced from natural gas, is used in many products, including adhesives, bonding agents, and solvents. HCHO reacts as an electrophile with the side-chains of arginine and lysine and the amino groups of RNA and DNA, and causes protein-protein, protein-DNA, and DNA-DNA cross-links. Therefore, it is suspected to be carcinogenic and a cause of sick-house syndrome.1–3 HCHO is also known as one of the major volatile organic compounds (VOCs) of air pollution and the World Health Organization has established an air quality guideline of 0.1 mg m–3. Utilization of houseplants for the removal of VOCs was first proposed by Wolverton et al.4 Although some houseplants were found to have relatively high ability to remove HCHO from the air,5 later studies revealed that the main organisms involved were not the plants themselves, but rather soil microorganisms symbiotically living with the plants.6 A trial to enhance the capacity for HCHO detoxification in plants was made by overexpressing an enzyme for HCHO oxidation, glutathione-dependent formaldehyde dehydrogenase.7–9 It is known that plants generally have a very limited capacity for assimilating gaseous HCHO,8 though HCHO is present as a natural metabolite in plants, mainly in forms bound with cofactors such as tetrahydrofolate and glutathione.9 On the other hand, some microorganisms are known to fix HCHO into their cell constituents when they produce it endogenously as a metabolite and when they are exposed to exogenous HCHO. The ribulose monophosphate (RuMP) pathway is one of the HCHO-fixation pathways found in microorganisms called methylo trophs, which utilize one-carbon compounds, such as methane and methanol, as the sole carbon source.10–12 The key enzymes of this pathway are 3-hexulose-6-phosphate synthase (HPS), which fixes HCHO to d-ribulose 5-phosphate (Ru5P) to produce d-arabino-3-hexulose 6-phosphate (Hu6P), and 6-phospho-3-hexulose isomerase (PHI), which converts Hu6P to fructose 6-phosphate (F6P).

The bacterial RuMP pathway and the plant Calvin-Benson cycle share common metabolic features: (i) both pathways fix a one-carbon unit to ribulose phosphate; (ii) the fixation reaction eventually yields F6P; and (iii) Ru5P is regenerated from F6P through rearrangement reactions. Based on these facts, we intended to introduce

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Abbreviations: BA, N6-benzyladenine; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; Gen, gentamycin; HCHO, formaldehyde; HPS, 3-hexulose-6-phosphate synthase; Hu6P, d-arabino-3-hexulose-6-phosphate; Km, kanamycin; MS, Murashige and Skoog; NAA, naphthalene acetic acid; NMR, nuclear magnetic resonance; Nos, nopaline synthase; 3-PGA, 3-phosphoglycerate; PHI, 6-phospho-3-hexulose isomerase; rbs, the gene for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; R5P, d-ribose 5-phosphate; rmpA, the gene for 3-hexulose-6-phosphate synthase; rmpB, the gene for 3-hexulose-3-phosphohexulose isomerase; Ru5P, d-ribulose 5-phosphate; RuBP, r-ribulose 1.5-bisphosphate; RuMP, ribulose monophosphate; Spe, spectinomycin; Su7P, sedoheptulose 7-phosphate; TP, transit peptide; TCA, trichloroacetic acid; TLC, thin layer chromatography; VOC, volatile organic compound; Xu5P, d-xylulose 5-phosphate
a HCHO-fixing reaction catalyzed by HPS and PHI into plants and to couple it with the RuMP regeneration reaction of the Calvin-Benson cycle. In the pathway designed, the CO₂-fixation and reduction steps in the cycle are bypassed by the HPS- and PHI-catalyzed reactions (Fig. 1).

In the present study, we produced transgenic Arabidopsis and tobacco plants that expressed the HPS- and PHI-encoding genes, \textit{rmpA} and \textit{rmpB} respectively, from a methyloîtrphic bacterium, \textit{Mycobacterium gastri} \textit{MB19,13) and targeted their products to the chloroplasts with artificially-added transit peptides.14) The resulting \textit{rmpA} and \textit{rmpB} were expressed in Arabidopsis thaliana (ecotype Columbia and \textit{A. tumefaciens} cv. Xanthi) was transformed essentially as described by Clough and Bent,17) with minor modifications. \textit{Arabidopsis thaliana} plants were grown in pots in soil, covered with a window screen, under constant light at 22 °C in a growth chamber. The \textit{rmpA} and \textit{rmpB} plant selection. Arabidopsis plants at the flowering stage were used in the experiments.

Materials and Methods

Plant materials. \textit{Arabidopsis thaliana} ecotype Columbia and \textit{Nicotiana tabacum} cv Xanthi were used in this study. Transgenic plants of T2 generation were used in the experiments.

Plasmid construction. The coding regions of the \textit{rmpA} (624 bp) and \textit{rmpB} (600 bp) genes were amplified by PCR from pUC118-rmpAB.13) For amplification of the \textit{rmpA} coding region, sense primer, 5'-GCA-TGCAGCTCAAGAGATGAGCCGCGGGATGG-3' (containing a BamHI site) were used. For amplification of the \textit{rmpB} coding region, sense primer 5'-GACTGCGGCAAAGCGAGAAGC-3' (containing a SphI site) and antisense primer 5'-GGAATCTCTAGAGTGCGGTGCGAC-3' (containing a BamHI site) were used. The amplified coding regions were subcloned into a TA cloning vector (Takara Bio., Ohtsu, Japan) to generate two plasmids, which were confirmed by digestion with \textit{BamHI} and \textit{SphI} enzymes. The resulting \textit{rmpA} and \textit{rmpB} coding regions in the two plasmids were confirmed.

The \textit{rmpA} coding region (about 624 bp) was isolated from pTA-rmpA and ligated into vector pUC-rbcS-SC15,16) to generate a new plasmid named pUC-rbcS-rmpA (about 5.4 kb). In the resulting plasmid, the \textit{rbcS} gene was inserted downstream of the transit peptide of the \textit{rbcS} gene and the core coding region of the \textit{rbcS} gene was removed. Similarly, the \textit{SphI-XbaI} fragment (about 600 bp) from pTA-rmpA was ligated into vector, pUC-rbcS-3C to generate a plasmid named pUC-rbcS-rmpB (about 5.2 kb). The pUC-rbcS-rmpA plasmid was then digested with \textit{HindIII} and \textit{BamHI}, and a 2.4-kb fragment containing the \textit{rbcS}-3C promoter and the \textit{rpmA}-coding regions was obtained. This fragment was subcloned into binary vector pPP211 at the \textit{HindIII} and \textit{BamHI} sites to generate the plant expression vector pPP221-rmpA, which carries a chimeric gene consisting of the \textit{rbcS}-3C promoter with its transit peptide, the \textit{rpmA}-coding region, and the nopaline synthase (Nos) terminator. Similarly, the \textit{rmpB} coding region (about 2.4 kb) from pUC-rbcS-rmpB was prepared and then subcloned into another binary vector, pPP221 at the \textit{HindIII} and \textit{BamHI} sites, generating another plant expression vector, pPP221-rmpB, which carries a chimeric gene, consisting of the \textit{rbcS}-3C promoter with its transit peptide, the \textit{rpmB}-coding region and Nos terminator. The two plant expression vectors were transferred into \textit{Agrobacterium tumefaciens} C58C1 (pMP90) by electroporation using a Gene Pulser (BioRad, Hercules, CA) with parameters 200 ohms and 2.5 kV/0.2 cm. \textit{A. tumefaciens} colonies were selected on LB-agar plates containing 100 mg/ml of spectinomycin (Spe).

Plant transformation. \textit{Arabidopsis} transformation was carried out essentially as described by Clough and Bent,17) with minor modifications. \textit{Arabidopsis thaliana} plants were grown in pots in soil, covered with a window screen, under constant light at 22 °C in a growth chamber. The \textit{A. tumefaciens} strain harboring the binary vector was grown in LB medium with Spe (100 μg/ml) and gentamycin (Gen, 25 μg/ml), until the culture reached an OD600 reading of 0.8–1.0. \textit{Arabidopsis} plants at the flower budding stage were infiltrated under vacuum with \textit{A. tumefaciens} cells that had been centrifuged and resuspended in 5% sucrose plus Silwet L-77 (0.005%) solution. Seeds were collected from the infiltrated plants and sown on agar medium containing kanamycin (Km, 50 μg/ml) or Gen (80 μg/ml) for transgenic \textit{rmpA} and \textit{rmpB} plant selection respectively. To generate double transformants, \textit{rpmA} and \textit{rpmB} transformants were reciprocally re-transformed with the \textit{rpmB} and \textit{rpmA} constructs. The double transformants were selected on agar medium containing both Km and Gen.

Tobacco \textit{(Nicotiana tabacum} cv. Xanthi) was transformed essentially by following the leaf disk co-cultivation protocol of Horsch et al.18) Co-cultivation was initiated by dipping leaf disks in an \textit{Agrobacterium} suspension, blotting them on sterile tissue paper, and incubating them for 2 d on Murashige and Skoog (MS) salts mixture medium containing naphthalene acetic acid (NAA, 0.21 μg/ml), α-naphthalenylacetic acid (BA, 0.02 μg/ml) and 3% sucrose for callus induction. The leaf disks were then transferred onto a medium containing antibiotics for transgenic plant selection, and NAA (0.105 μg/ml), BA (0.5 μg/ml), and 3% sucrose for shoot induction. Carbenicillin was included in the medium (500 μg/ml) to inhibit \textit{Agrobacterium} growth.
As for Arabidopsis, tobacco double transformants were generated by re-transformation. Tobacco seedlings were tested for antibiotic resistance by germinating them on MS medium containing 1% sucrose and antibiotics.

**Growth and maintenance of transgenic plants.** For aseptic growth of Arabidopsis plants, seeds were surface sterilized, cold treated at 4–6°C for 1–2 d, and germinated on a solidified medium containing agar (1%), MS/G salts (1/2 Murashige and Skoog salts), 1/2 Gamborg's B5 salts, 0.05% MES (pH 5.7), and 1% sucrose, with and without antibiotics. The seedlings were grown in a growth chamber at 22°C under constant light. Resistant seedlings were transplanted to Japanese “Green Soil” (Sanwa Press, Tsu, Mie, Japan) for propagation, and were grown under constant light at 22°C in a greenhouse. For aseptic growth of tobacco, seeds were surface sterilized and germinated on a solidified medium containing agar (0.8%), MS salts (pH 5.7), and sucrose (3%), with and without antibiotics, in a growth chamber at 25°C under constant light. For propagation of tobacco, the selected seedlings were transplanted to soil and grown under constant light at 25°C in a greenhouse.

**Northern analysis.** Transgenic plants were first selected on agar medium with antibiotics and then grown for 2 weeks on medium without antibiotics to obtain vigorous growth. Total RNA was isolated from the seedlings using TRIZol Reagent (Invitrogen, Carlsbad, CA) as described by the manufacture. The coding regions of the rmp4 and rmp5 genes were used as probes. In Northern blot analysis, total RNA was fractionated by electrophoresis on 1.2% agarose gels containing 6% v/v formaldehyde, blotted onto nylon membranes, and hybridized with [32P]-labelled DNA probes.

**Western analysis and measurement of enzyme activity.** Arabidopsis and tobacco seedlings (1–2 g), grown on agar medium or soil, were harvested and homogenized in 10 volumes w/v of protein extraction buffer (50 mM phosphate buffer pH 7.5, 10% glycerol, 5 mM MgCl2, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfenyl fluoride, 2 mM EDTA, and 10% w/v insoluble polyvinylpyrrolidone). Each extract was centrifuged at 15,000 x g for 20 min at 4°C. The supernatant was collected and the protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard. Intact chloroplasts were isolated from Arabidopsis leaves as described by Tribouh et al., with modifications. The chloroplasts were disrupted and centrifuged to obtain the soluble protein fraction. For Western blot analysis, the proteins were separated in SDS–PAGE gel (15%), and then transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were first treated with rabbit antibodies raised against HPS or PHI from Methylomonas aminofaciens 77a, and then with a goat antibody raised against rabbit IgG which had been conjugated with peroxidase.

**Enzyme activity** was determined spectrophotometrically by monitoring the HCHO-dependent production of NADPH at 340 nm, as described by Kata. The enzyme reaction was performed at 30°C in an assay mixture containing 50 mM potassium phosphate buffer pH 7.5, 2.5 mM MgCl2, 2.5 mM Ribose 5-phosphate (R5P), 2.5 mM NADP+, 10 U phosphoriboisomerase, 10 U phosphoglucomutase, and 10 U glucose phosphate dehydrogenase with 10 U PHI or HPS, 2.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfenyl fluoride, 2 mM EDTA, and 0.05% MES (pH 5.7), and 1% sucrose, with and without antibiotics, in a growth chamber at 22°C under constant light. For propagation of tobacco, the selected seedlings were transplanted to soil and grown under constant light at 25°C in a greenhouse.

For measurements of aseptic growth of tobacco, seeds were surface sterilized and germinated on a solidified medium containing agar (0.8%), MS salts (pH 5.7), and sucrose (3%), with and without antibiotics, in a growth chamber at 25°C under constant light. For propagation of tobacco, the selected seedlings were transplanted to soil and grown under constant light at 25°C in a greenhouse.

**Aseptic growth of tobacco.** Tobacco seedlings were selected and grown on MS medium for 2 weeks. The seedling s (0.25 g) were collected and submerged in 10 ml of water containing various concentrations of HCHO in a 50 ml-flask for 30 h at 20°C under light. Samples of the HCHO solutions were then taken for measurement of the HCHO concentration. A mixture of 50 μl of sample solution, 950 μl of H2O and 1 ml of freshly prepared Nash reagent (15.0 g of ammonium acetate dissolved in 99.5 ml of H2O, 0.3 ml of acetic acid, and 0.2 ml of acetone) was incubated at 30°C for 30 min. The HCHO concentrations were measured at 410 nm with pure HCHO as standard.

**Tracer experiments.** For long-term tracer experiments, Arabidopsis seedlings were selected and grown on MS medium for 2 weeks. The seedlings (0.25 g) were collected and submerged in a solution containing 5 mM KOH2O, 1 mM HCHO, 0.1% MES (pH 5.7), and [14C]HCHO (Sigma, St. Louis, MO, 2.8 μCi) and incubated under constant light at 25°C overnight. After incubation, the plants were washed with cooled sterile water 4–5 times to remove free [14C]HCHO from the surfaces. They were homogenized in liquid-nitrogen and extracted with 10% trichloroacetic acid (TCA). The insoluble fraction was obtained by centrifugation and washed once with 5% TCA, twice with 50% ethanol, and once with 100% ethanol by filtration. The radioactivity in the insoluble fraction was measured using a Clear-sol scintillation cocktail in a liquid scintillation counter.

In short-term tracer experiments, seedlings (1 g of fresh weight) were immersed in a 20 ml solution containing 5 mM KOH2O, 1 mM HCHO, 0.1% MES (pH 5.7), and [14C]HCHO (Sigma, 2.0 μCi) and incubated under constant light at 25°C for 50 min. After incubation, the plants were washed with cooled sterile water 4–5 times to remove free [14C]HCHO from the surfaces. The soluble metabolites in the plants were extracted with 80% v/v hot ethanol, then dried, then dissolved in methanol (100 μl). A portion of the sample was subjected to radioactive measurement, and the rest of the sample was analyzed by thin-layer chromatography (TLC). One-dimensional TLC was performed on thin-layer cellulose plates (Merck no. 1.05716) using butanol-propionic acid-water (equal parts of butanol-water, 370:25 v/v, and propionic acid-water, 18:22 v/v) as the solvent system as described by Pedersen et al.

[14C]HCHO labeling experiments, the plants (1.5 g) were immersed in a solution containing 5 mM KOH2O, 2 mM [14C]HCHO (Cambridge Isotopes Laboratories, Andover, MA) and 0.1% MES (pH 5.7), and incubated under constant light at 25°C for 5 or 10 min. After incubation, the plants were washed with cooled sterile water 4–5 times to remove free [14C]HCHO from the surfaces. They were homogenized in liquid nitrogen, then 2 ml of 100 mM potassium phosphate buffer (pH 7.4) was added and the mixture was boiled for 3 min. Cellular debris was removed by centrifugation. The supernatant was frozen, lyophilized, and resuspended in 0.5 ml of the phosphate buffer containing 5% D2O, and placed in a 5-mm NMR tube.

**NMR analysis.** [14C]NMR analysis was performed (at Yunnan University, China) on a Bruker DRX 500-MHz instrument (Karlsruhe, Germany). Broad-band decoupling experiments were carried out for qualitative analysis of NMR spectra for extracts from the plants labeled for 5 min with [14C]HCHO. The acquisition parameters included a 6.8 μs (30°) pulse with broad-band proton decoupling, a spectral width of 32,679 Hz, an acquisition time of 0.5 s, and a decay time of 1.2 s. The sample temperature was maintained at 25°C, and 32,000 data points were acquired for each sample. Scans (n = 1,200) were acquired for each sample, and a line broadening of 4 Hz was used in the processing of free induction decay.
Results

Expression of bacterial HCHO-assimilation enzymes in Arabidopsis chloroplasts

Arabidopsis thaliana was transformed with two kinds of DNA constructs containing the rmpA and rmpB genes (Fig. 2A). Both of them were placed under the control of the tomato rbcS-3C promoter with its transit peptide sequence following Miyagawa et al. Transgenic lines (at least 20 of each) containing rmpA (named A1–A20), rmpB (B1–B20), or both rmpA and rmpB (AB1–AB20) were obtained, as described in “Materials and Methods.” Plants transformed with one of the two empty vectors, pFPZP211 or pFPZP221, were produced as controls. One line of the former transformants, named CK, was used as a control in this work. All the transformants appeared to be indistinguishable from wild-type plants in the vegetative and reproductive stages when grown either on an antibiotic-free agar medium or on soil. Seeds of each plant line were collected, and T2 generation plants were used in further experiments.

Expression of the rmpA and rmpB genes in the transgenic plants was confirmed by Northern blot analysis (Fig. 2B, C), and by Western blot analysis using rabbit antibodies against the HPS and PHI proteins (Fig. 2D). The HPS and PHI proteins were found only in the extracts of chloroplasts purified by subcellular fractionation from transformants carrying the rmpA and/or rmpB genes respectively. Correct removal of the transit peptide in the chloroplasts was indicated by the molecular sizes of HPS (21 kDa) and PHI (25 kDa), which were identical to those of the original proteins. These results indicate that the HPS and PHI proteins were successfully expressed and targeted to the chloroplasts. A coupled spectrophotometric method was used to assay HPS and PHI activities in the leaf extracts.

Quantitative analysis of \([^{13}C]\)-enriched F6P and G6P in extracts from the plants labeled for 10 min with \([^{13}C]HCHO\) were accomplished through inverse-gated decoupled experiments with a long decay. The parameters included a 2.5-μs (45°) pulse, a spectral width of 31, 250 Hz, an acquisition time of 0.5 s, and a delay time of 20 s. Decoupling was achieved using the composite pulse Waltz-16 only during the acquisition period. The sample temperature was maintained at 25°C, and 32,000 data points were acquired for each sample. Twelve hundred scans were acquired for each sample, and a line broadening of 4 Hz was used in the processing of free induction decay.

Resonances were assigned based on the chemical shifts of authentic samples and by comparing the observed chemical shifts with previously published values. Broad-band decoupling experiments were used to cross-check the quantities estimated from the inverse-gated experiments. The peaks were calibrated relative to \([^{13}C]HCHO\) sealed in a capillary and inserted in the sample tube.
and it revealed easily detectable levels of enzyme activity in the transgenic plants. The HPS specific activities in the extracts from CK, A1, B1, AB1, and AB2 lines were 0, 0.24, 0, 0.28, and 0.20 μmol/min/mg protein respectively, and the PHI-specific activities from the CK, A1, B1, AB1, and AB2 lines were 0, 0, 0.20, 0.16, and 0.18 μmol/min/mg protein respectively.

Enhanced tolerance of transgenic A. thaliana planted on agar media containing HCHO

Since HCHO is highly toxic to plants, we speculated that transgenic plants engineered with the HCHO-fixation pathway would become tolerant to higher concentrations of HCHO than control plants. The HCHO tolerance of transgenic A. thaliana was investigated on agar medium containing HCHO. After germination, Km- and/or Gen-resistant seedlings of the A1, B1, AB1, and the CK transgenic plants were grown aseptically on agar medium without antibiotics for 7 d and then transferred to agar media containing various concentrations of HCHO. At 4 mM HCHO, no difference was observed in growth among the seedlings (Fig. 3A). At 8 mM HCHO, the CK and B1 plants did not grow, and they showed chlorosis and wilting. On the other hand, about 50% of the A1 plants survived. This indicates that the production of HPS in chloroplasts enhanced HCHO tolerance to some extent. No growth inhibition was observed in the AB1 transgenic plants (Fig. 3B). In the presence of 10 mM HCHO, only the AB1 transgenic plants survived (Fig. 3C). Thus co-expression of HPS and PHI endowed the plants with the ability to detoxify HCHO more effectively than individual expression of HPS or PHI.

Enhanced tolerance and uptake capacity of transgenic A. thaliana to gaseous HCHO

To determine whether the AB transgenic plants would also show higher tolerance and uptake capacity as to gaseous HCHO, the AB1, AB2, and CK lines, which had been grown separately in sealed boxes, were exposed to HCHO evaporated from a micro test tube (0.5 ml) containing HCHO solution (37%, 50 μl) for about 3 weeks (Fig. 4). Within 1 week after the start of exposure, chlorosis and wilting occurred in the CK plants, but not in the AB1 or AB2 plants, indicating enhanced tolerance of the double transformants to gaseous HCHO. After 3 weeks, the covers of the plant boxes were removed and quickly replaced with covers equipped with HCHO dose-monitoring tubes in order to determine roughly the gaseous HCHO levels remaining in the boxes. As shown in the lower part of Fig. 4, the concentrations were found to be less than 2 ppm for the AB1 and AB2 lines, but more than 20 ppm for the CK plants. These results indicate that the HCHO assimilation pathway strongly enhanced not only the tolerance of the transgenic AB plants to exogenous HCHO, but also their ability to take up and eliminate gaseous HCHO.

Removal of exogenous HCHO by transgenic A. thaliana

To test the ability of the transgenic plants to remove exogenous HCHO, experiments were performed as described by Achkor et al.7) The seedlings of AB1 and CK plants were suspended in a solution (0.2% MES,
5 mM KHCO₃, pH 5.7) containing various concentrations (10, 8, and 5 mM) of HCHO, and shaken for 30 h under light. The concentrations of HCHO decreased to 67% and 80% of the initial concentration of 10 mM, for AB1 and CK1 respectively. In the same way, decreases to 30% (AB1) and 69% (CK1) of the initial concentration of 8 mM, and the decreases to 9% (AB1) and 46% (CK1) of the initial concentration of 5 mM were observed. These results indicate that at lower initial concentrations, 5 and 8 mM, the amounts of HCHO eliminated by AB1 and CK1 were about 50 µmol and 25 µmol respectively. Thus the apparent capacity for HCHO removal of AB1 was about 2-fold higher than CK1. However, it must be kept in mind that the elimination of HCHO measured here might have been due not only to cellular metabolism (degradation and assimilation) but also to HCHO-adduct formation with various cell constituents.

Augmented HCHO assimilation in transgenic A. thaliana

To demonstrate the assimilation of HCHO through the engineered HCHO-fixation reaction in the AB1 transgenic plants, a tracer experiment with [¹⁴C]HCHO was conducted under the conditions described for the above experiment, except that the initial HCHO concentration was 2 mM. As a precaution, non-radioactive KHCO₃ was included in the solution to dilute out the [¹⁴C]CO₂ that can be produced from [¹⁴C]HCHO during incubation. After 18 h of incubation under light, the seedlings were washed and then ground in the presence of TCA. The radioactivity of the TCA-insoluble fraction was about 10-fold higher in the AB1 transgenic plants than in the CK control plants (Fig. 5A). Since HCHO-adducts are supposed to be decomposed by TCA-treatment, the radioactivity in this fraction can be ascribed to high-molecular-weight cell constituents. When short-term incubation (1 h) was carried out and the soluble fraction was prepared by hot-ethanol extraction, the radioactivity in this fraction was about 2-fold higher for the AB1 plants than for the CK controls (Fig. 5B). Cellulose TLC analysis of this fraction indicated that the radioactivity was incorporated into a discrete number of metabolite groups (Fig. 5C). Among the three major spots in Fig. 5C, spot 1 had mobility similar to those of sugar monophosphates. Although no characterization of the spots was carried out, these results indicate that HCHO assimilation capacity was augmented in the transgenic plants as compared with the control plants.

Functioning of the engineered HCHO fixation pathway

To confirm that the HCHO fixation pathway was indeed established as a bypass of the Calvin-Benson cycle by overexpression of HPS and PHI in the transgenic plants, the [¹⁴C]nuclear magnetic resonance (NMR) technique was used to detect the labeled intermediates in the Calvin-Benson cycle after the A1, B1, AB1, and CK plants were incubated with [¹⁴C]HCHO for 5 min as described above. Labeled metabolites were extracted from the plants with phosphate buffer and then subjected to [¹⁴C]NMR analysis. The [¹⁴C]NMR spectra are shown in Fig. 6. An extract of wild-type (WT) Arabidopsis plants without [¹⁴C]HCHO treatment was used to monitor the background [¹⁴C]NMR signal levels of the intermediates which is shown in Fig. 6E. The resonance signal peaks corresponding to the initial HCHO fixation product, [¹⁴C]F6P, with chemical shifts at 63.77, 64.05, and 64.76 ppm were observed in the AB1 spectra (Fig. 6A), but no such signals were detected in the spectra of the CK (Fig. 6B), A1 (Fig. 6C), or B1 (Fig. 6D) extracts. Signal peaks corresponding to the other intermediates, including [¹⁴C]Ru5P, [¹⁴C]ribose 5-phosphate (R5P), [³¹⁴C]sedoheptulose 7-phosphate (Su7P), [¹⁴C]ribulose 1,5-bisphosphate (RuBP), [³¹⁴C]3-phosphoglycerate (3-PGA), and [¹⁴C]xylulose 5-phosphate (Xu5P) with chemical shifts at 171.53, 98.77, 73.56, 68.46, 66.77, and 63.36 ppm respectively, also appeared in the AB1 spectra (Fig. 6A), no such signals were detected in the spectra of the CK (Fig. 6B), A1 (Fig. 6C), or B1 (Fig. 6D) extracts. Two specific resonance signal peaks with chemical shifts at 72.18 and 72.3 ppm, which appeared only in the spectra of AB1 (Fig. 6A) and A1 (Fig. 6C) extracts, might correspond to [¹⁴C]Hu6P. In addition, part of the [¹⁴C]F6P might have been converted to...
[1-13C]glucose 6-phosphate (G6P), which appeared as two strong resonance signal peaks, at 92.83 and 96.66 ppm in the AB1 spectra. G6P and F6P are known to be readily inter-convertible by the action of G6P isomerase. There were some background signals of [1-13C]F6P and [1-13C]G6P in the spectra of WT extract (Fig. 6E), presumably due to the natural abundance of 13C in atmospheric CO2. Similar levels of signal were also detected in the extracts of CK (Fig. 6B), A1 (Fig. 6C), and B1 (Fig. 6D). Hence, quantitative [13C]NMR analysis was performed with extracts from the AB1 and CK plants incubated with [13C]HCHO for 10 min to determine whether the [1-13C]F6P and [1-13C]G6P contents in the AB1 extract truly increased. The results indicated that the [1-13C]F6P and [1-13C]G6P levels in the AB1 extract were almost 3-fold higher than those in the CK extract (Fig. 7). This indicates that [13C]HCHO was rapidly fixed to F6P owing to efficient functioning of the installed HCHO-fixation pathway, and subsequently was incorporated into the expected positions of the intermediates in the Calvin-Benson cycle. The data indicates that [13C]HCHO was indeed assimilated in the engineered HCHO fixation pathway, which functioned as a bypass of the cycle.

Enhancement of HCHO tolerance in transgenic tobacco by co-expression of HPS and PHI

To determine whether the strategy would work generally in other plants, the rmpA and rmpB construct was introduced into tobacco. Ten lines of rmpA transformants (A1–A10) and 15 lines of rmpB transformants (B1–B10), and ten lines of double-gene transformants (AB1–AB10) were obtained. Control lines (CK) were transformed with the empty vector pPZP211. Expression of the designated genes in the tobacco transformants was confirmed by Northern blot analysis, Western blot analysis, and enzyme assay in the same manner as described above for Arabidopsis (data not shown). These transgenic tobacco plants were treated with HCHO, and the results for representative lines (A1, B7, AB7, and CK) are shown in Fig. 8. Shoots of these lines were cut and transferred to agar medium containing 10 mM HCHO and grown for 2 weeks. Control and B7 plants (Fig. 8A and B) were severely damaged by HCHO, and root growth was almost completely inhibited. The A1 shoots were damaged by HCHO (Fig. 8C), but not as severely as the control and B7 shoots. A1 root growth

Fig. 6. [13C]NMR Analysis of the Metabolites of HCHO Assimilation by Transgenic Arabidopsis Plants.
Complete [13C]NMR spectra (left) and expanded regions of interest (right) of leaf extracts of AB1 (A), CK (B), A1 (C), and B1 (D) treated with [13C]HCHO for 5 min, as well as leaf extract of WT without [13C]HCHO treatment (E). Experiments were carried out in the manner described in Fig. 5, except for the use of [13C]HCHO instead of [14C]HCHO. For details, see “Materials and Methods.” The transgenic lines are described in the legend to Fig. 2. Peak assignments are as follows: Ref, reference; Ru5P, [1-13C]Ru5P; Ri5P, [1-13C]Ri5P; Su7P, [3-13C]Su7P; RuBP, [1-13C]RuBP; 3-PGA, [3-13C]3-PGA; F6P, [1-13C]F6P; Xu5P, [1-13C]Xu5P; Hu6P, [1-13C]Hu6P; G6P, [1-13C]G6P.

Fig. 7. Quantitative Analysis of [1-13C] F6P and [1-13C] G6P in Cell Extracts from the AB1 and CK Seedlings after Incubation with [13C] HCHO (2 mM) for 10 min.
Experiments were carried out as described in “Materials and Methods.” The transgenic lines are described in the legend to Fig. 2.
was inhibited, but not completely, by HCHO. The AB7 shoots were not significantly damaged by HCHO (Fig. 8D), and they produced strong root systems and thus exhibited vigorous growth. These results were similar to those observed with transgenic Arabidopsis, indicating that the RuMP pathway functioned as expected in the transgenic tobacco.

In order to demonstrate a synergistic effect of the co-expression of HPS and PHI on HCHO tolerance, it is preferable to prepare lines of double gene transformants by cross pollination between the single gene transformants rmpA (A1) and rmpB (B7). In the resulting progeny, the dosage of each gene cannot exceed the dosage of either parent. Line B7 was pollinated with line A1, and cross progenies A × B were obtained. A line named A1 × B7, which must have had both genes as determined by its tolerance to the two antibiotics for selection, was used in the experiment. Seeds of line A1, line B7, and line A1 × B7 were sown on agar medium and grown for 3 weeks, and then a microtube (0.5 ml) containing 20 μl of 37% w/v HCHO was placed in each box. Transgenic plants grown without exposure to HCHO are also shown as controls. Photographs were taken after 2 weeks of exposure.

Discussion

The present study demonstrates, for the first time, the integration of a bacterial HCHO fixation pathway into the Calvin-Benson cycle in transgenic plants. It is quite feasible that this pathway functions in plant leaves, because: (i) Gaseous HCHO is assumed to diffuse into the leaves through the stomata efficiently, as is the case for NO₂, (ii) the large metabolic flow of the Calvin-Benson cycle provides for a sufficient supply of the acceptor, RuMP and prompt removal of the product, F6P, and (iii) no additional energy is required. Therefore the engineered HCHO fixation pathway can be considered a bypass of the CO₂-fixing reaction in photosynthetic plants (see Fig. 1).

Overexpression of a glutathione-dependent HCHO dehydrogenase (FALDH) has been reported to enhance HCHO elimination in Arabidopsis. We produced the same transgenic Arabidopsis plants reported by that group and compared their tolerance to HCHO. In contrast to our transgenic plants expressing HPS and PHI, the FALDH overexpressing plants did not grow on an agar medium containing 6 mM HCHO (data not shown), indicating that no or very weak tolerance to HCHO can be conferred by the introduction of this gene. More recently, an adverse effect of FALDH overexpression on plant growth was reported by the same research group (ref. 7). This FALDH system requires, in addition to the cofactor glutathione, regeneration systems for the reduced glutathione and NAD⁺, whereas the RuMP pathway for HCHO fixation described here does not require any additional energy (it is exergonic) or cofactors, and the growth of the transgenic plants was not inhibited in any way. Furthermore, the regeneration pathway of Ru5P is inherent in plants. Hence we believe that the RuMP pathway is a more suitable HCHO elimination system in plants.

Our preliminary experiments with larger tobacco (about 90 cm in height) in 300-liter chamber indicated that the rate of HCHO absorbance was about 20% faster.
for the transgenic tobacco (AB) than the wild-type plants.\textsuperscript{27} If this strategy can be applied to indoor houseplants, the resulting genetically modified plants can be used to maintain a healthy household environment, and may be more favorably accepted by the public than genetically engineered foods. It is also noteworthy that there are no direct routes to produce biomass from reduced C\textsubscript{1} compounds such as methane (a greenhouse gas), methanol, or HCHO in plants. Therefore, the RuMP-plant system may provide a novel photosynthetic platform for the assimilation of these natural gas-derived C\textsubscript{1}-compounds and biogas emitted from various plants.

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