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Original Paper

Efficient assimilation of sulfide by transgenic rice plants over-expressing a rice cysteine synthase

Yube Yamaguchi[a], Tatsuo Nakamura[b], Yuko Tatsumi, Kimiyo Nakamura, Hiroshi Sano*

Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Nara 630-0192, Japan

* E-mail: sano@gtc.naist.jp Tel: +81-743-72-5650 Fax: +81-743-72-5659

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Abstract Hydrogen sulfide is a major environmental pollutant, highly toxic to living organisms at high concentrations. Even at low concentrations, it causes an unpleasant odor from wetlands, especially from wastewater. Plants can utilize hydrogen sulfide as a sulfur source to synthesize cysteine, which then serves as the principal substrate for synthesis of other sulfur containing compounds including glutathione and methionine. It was thus feasible to use aquatic plants, which possess high potential for sulfur assimilation, to remove hydrogen sulfide from the wetland. To this end, we have generated transgenic rice plants over-expressing cysteine synthase, a key enzyme in the sulfur assimilation pathway, and evaluated their capacity for sulfur uptake on hydrogen sulfide treatment. The obtained transgenic plants exhibited 3-fold elevated cysteine synthase activity, and incorporated more hydrogen sulfide into cysteine and glutathione than their wild type counterparts upon exposure to a high level of hydrogen sulfide. These observations suggest that over-expression of cysteine synthase in aquatic plants is a viable approach to remove hydrogen sulfide from polluted environments.

Key words: Cysteine synthase, hydrogen sulfide, *Oryza sativa*, phytoremediation.

Hydrogen sulfide can cause serious damage to living organisms, such as eye irritation and apnea at high concentrations (70–700 $\mu g\,l^{-1}$) in the air. Even at low concentrations (4.2–7 $\mu g\,l^{-1}$), it causes a pervasive offensive odor (National Research Council 1979). Hydrogen sulfide is produced both naturally and artificially as a result of human activity. In nature, it is produced through non-specific anaerobic bacterial reduction of sulfate and sulfur-containing organic compounds, frequently occurring in lakes and wetlands (Hill 1973). When these places are polluted with wastewater, the amount of emitted hydrogen sulfide is readily increased. Levels of sulfide vary considerably and concentrations of hydrogen sulfide (as sulfide sulfur) in wastewater are reported to range from 3.1–5.1 $mg\,l^{-1}$ (Parvinen and Lajunen 1994). In the United States, the total sulfide level in one sample from the Mississippi River was approximately 0.92 $mg\,l^{-1}$, and 1.6 $mg\,l^{-1}$ has been noted for pond and well water in St Paul, Minnesota (International Programme on Chemical Safety 2003).

Plants are able to incorporate hydrogen sulfide through the sulfate assimilation pathway, the cysteine end-product then being further utilized for synthesis of other sulfur containing compounds, including glutathione, methionine and phytochelatins (Leustek et al. 2000; Yamaguchi and Sano 2001). Hydrogen sulfide is generally synthesized from sulfate, which is present in plant cells, but gaseous hydrogen sulfide from the air can also be utilized by plants (Schmidt and Jäger 1992).

Since a high level of sulfite and sulfide is toxic to most plants, attempts have been made to increase the capacity for cysteine biosynthesis, in the hope of conferring tolerance to these substances. Various types of transgenic plants have thereby been generated using genes involved in the sulfate assimilation pathway, and their physiological features evaluated (Sirko et al. 2004). For example, transgenic tobacco plants over-expressing cysteine synthase from wheat (Youssefian et al., 1993; Youssefian et al. 2001) or spinach (Saito et al. 1994) showed an increased level of cysteine and glutathione in leaves, along with increased tolerance to hydrogen sulfide or sulfite. Water spinach (*Ipomea aquatica*) expressing adenosine phosphosulfate reductase, a key enzyme in the initial step of sulfur assimilation pathway, exhibited elevated sulfate assimilation ability (Sakulkoo et al. 2005). Some transgenic plants also demonstrated tolerance to heavy metals such as cadmium and certain oxidative stresses, probably due to synthesis of phytochelatins, the major component to detoxify cadmium in plants (Harada et al. 2001; Kawashima et al. 2004; Domingues-Solis et al. 2001; Youssefian et al.

Present addresses: [a] Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA; [b] Faculty of Environmental Information, Yokohama National University, Kanagawa 240-8501, Japan

This article can be found at http://www.jspcmb.jp/

2001; Sakulkoo et al. 2005).

Hitherto, research on detoxification of hydrogen sulfide has been focused solely on terrestrial dicotyledonous plants, with the long-range aim of generating sulfide-resistant plants. However, environmental pollution due to emission of hydrogen sulfide from eutrophicated wetlands has become a problem requiring urgent measures. For this purpose we have generated transgenic rice plants over-expressing a rice cytosolic cysteine synthase. Here we report that they indeed show an elevated potential for uptake of hydrogen sulfide from the environment.

Materials and methods

Construction of binary vector and transgenic rice plants

An EST clone with accession no. D47342 encoding the rice cytoplasmic cysteine synthase gene (*rcs1* accession no. AF073695) was obtained from the MAFF DNA Bank (National Institute of Agrobiological Sciences). The cDNA fragment was recovered after digestion with *Sal*I and *Sac*I, and then cloned into the pGEM-7Zf, digested with *Xho*I and *Sac*I. The resulting plasmid was transformed into *Agrobacterium tumefaciens* EHA101. Rice (*O. sativa* cv. Kinuhikari) transformation was essentially performed as described (Hiei et al. 1994).

Chemical treatments

Homozygous transgenic lines were selected by culturing seedlings of T_1 through T_3 generations of transgenic rice (see the following section) on MS medium containing 3% sucrose and 50 mM hygromycin. Surviving plantlets were transferred to soil, and grown under greenhouse conditions. To analyze the effects of the transgene, seedlings of wild type and transgenic (T_3 generation) plants (*Oryza sativa* cv Kinuhikari) were germinated and grown on soil. Hydrogen sulfide treatment was performed as follows. The youngest fully expanded leaves were cut into 4 cm lengths, and one side was soaked in 2.5 ml of tap water in a 25 ml bottle. Sodium sulfide (Na_2S) was added to the tap water, then the bottle was tightly sealed quickly. The final concentration of sulfide sulfur was 10 mg l^{-1}.

Isolation and analysis of DNA and RNA

Genomic DNA was isolated as described (Nakamura et al. 1999). Twenty micrograms of genomic DNA were digested with *Xba*I, size-fractionated by electrophoresis on 0.8% agarose gels, and blotted onto nylon membranes (Nakamura et al. 1999). Total RNA was isolated from leaves by the hot phenol method (Verwoerd et al. 1989) and 10 μg aliquots were fractionated on 1% agarose gels containing formaldehyde using 20 mM 3-(*N*-morpholino)propanesulfuric acid as a running buffer. The gels were blotted onto nylon membranes, which were then subjected to hybridization (Yamaguchi et al. 2000) using *rcs*1 cDNA as the probe.

Measurment of cysteine synthase activity

Rice leaves were ground using a Polytron (Brinkman Instruments, Wesbury, NY) in 1 ml of extraction buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, pH 7.5, and centrifuged at 12,000 *g* at 4°C for 30 min. Resulting supernatants were used as crude protein extracts. Cysteine synthase activity was determined by the method described earlier (Rolland et al. 1992) with modifications. The crude protein (0.3 μg) was added to a 100 μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM *O*-acetylserine, 2 mM Na_2S and 50 μM pyridoxal 5′-phosphate. The reaction was stopped by adding 20% (v/v) trichloroacetic acid after incubation at 30°C for 15 min, and the precipitated proteins were removed by centrifugation. Ninhydrin solution (300 μl, comprising 220 μl concentrated acetic acid, 80 μl concentrated HCl and 0.5 mg ninhydrin) was added to the reaction mixture, followed by boiling at 100°C for 10 min, and rapid cooling. The L-cysteine content was determined spectrophotometrically at A560 after adding 550 μl 95% ethanol.

Measurement of cysteine and glutathione contents

The amounts of cysteine and glutathione were estimated by HPLC as described (Noctor and Foyer 1998). Leaves (10 mg) were ground in 0.1 ml of grinding solution containing 0.1N HCl and 1 mM EDTA with liquid nitrogen using a mortar and pestle, and centrifuged at 10,000 *g* for 30 min at 4°C. For derivatization, 20 μl dithiothreitol was added to 200 μl of extract supernatant. Sufficient amounts of 0.5 M *N*-cyclohexyl-2-aminoethanesulfonic acid (pH 9.3 with NaOH) were added to bring the mixture to pH 8.0 and 20 μl monobromobimane (30 mM in acetonitrile) was immediately added. The mixture was rapidly mixed and derivatization allowed to proceed at room temperature in the dark. After 15 min, 0.8 ml 10% acetic acid was added and the mixture placed on ice for 5 min. Following centrifugation for 30 min at 10,000 *g* and 4°C, the supernatant was transferred into glass amber tubes and loaded into an autosampler tray. The injection volume was 10 μl. Monobromobimane derivatives were separated at room temperature on a TSK gel ODS-80Ts (4.6×150 mm, TOSOH, Tokyo) with a linear gradient from 100% buffer A (10% methanol, 0.25% acetic acid, pH 4.3 with NaOH) to 70% buffer A : 30% buffer B (90% methanol, 0.25% acetic acid, pH 4.3 with NaOH)

in 30 min (1 ml min^{-1}). Eluted compounds were detected fluorimetrically with excitation at 384 nm and emission at 462 nm.

Results and discussion

Generation of transgenic rice

Transgenic rice plants were successfully produced, in which the rice cytosolic cysteine synthase gene, *rcs*1, was over-expressed under control of the CaMV 35S promoter. Among 19 T_0 plants initially obtained, 8 were selected and grown to maturity. Features of mature plants were apparently the same as in the wild type controls, showing similar stem lengths and leaf numbers (Figure 1 shows two representative lines). This indicated no detrimental effects of the transgene. To obtain homozygous plants, T_2 seeds of the 8 lines were selected on medium containing hygromycine, and segregation ratio for resistance vs. sensitive to the drug was observed. Lines #2, #12, #13 and #14 apparently showed no segregation, suggestive to homozygotes (data not shown).

Transgene analyses

The copy number of integrated gene was examined by Southern hybridization. Lines #2, #12 and #13 showed a clear single signal in addition to signals present in the wild-type genome (Figure 2A, indicated by the open arrowhead), suggesting the lines to possess one copy of the transgene. Line #14 exhibited three additional signals, indicating the presence of three copies of the transgene (Figure 2A, indicated by the closed arrowhead). Expression of the transgene was then examined. Total RNA was extracted from leaf tissues and subjected to Northern hybridization assay. Results showed that transgenic lines #2, #12 and #13 constitutively accumulated high amounts of *rcs*1 mRNA, while wild type and transgenic line #14 scarcely expressed *rcs*1 (Figure 2B). The absence of transcripts in the latter is consistent with previous observations showing multi-copy transgenes are often transcriptionally inactive (Kooter et al. 1999).

Properties of transgenic lines

Whether or not these transcripts were correctly translated was examined by estimating the enzymatic activity of *O*-acetylserine(thiol)lyase, the product of *rcs* gene. When crude extracts from each transgenic lines were subjected to activity assays, all 3 lines (#2, #12 and #13), which expressed the transgene, showed an elevated activity up to 3-fold in comparison with the control wild type plant (Figure 3A). In contrast, the activity in line #14 did not differ from that of the control, being consistent with the absence of transgene transcripts. Based on these findings, we selected lines #12 and #13 for further experiments.

Figure 1. Phenotye of transgenic rice over-expressing the *rcs*1 gene. Wild type and transgenic plants were grown to maturity in a green house, and representative lines (#12; right and #13; middle) and wild type (WT; left) were photographed.

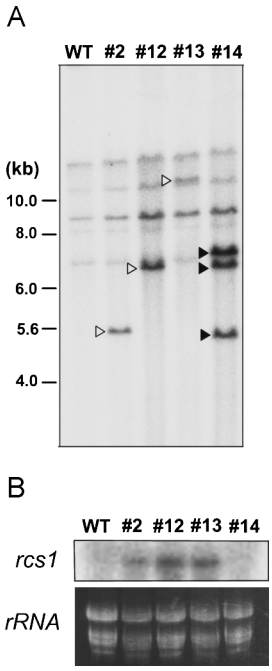

Figure 2. Transgene analyses. (A) Southern blot analysis of the transgene in the indicated transgenic lines. The introduced gene is indicated by the open arrowheads in lines #2, #12 and #13, and by the closed arrowhead for line #14. As a control, the wild type (WT) hybridization pattern is shown. (B) Northern hybridization analyses of *rcs1* transcripts (*rcs*1) in the indicated transgenic lines and wild type controls (WT). As a loading control, rRNA was used. Hybridization was performed using *rcs*1 cDNA as a probe.

Since the major compounds containing non-protein sulfur in plant cells are cysteine and glutathione, their contents were measured by HPLC using leaves from three month-old T_3 plants grown in a green house. Both wild type and transgenic plants contained nearly the

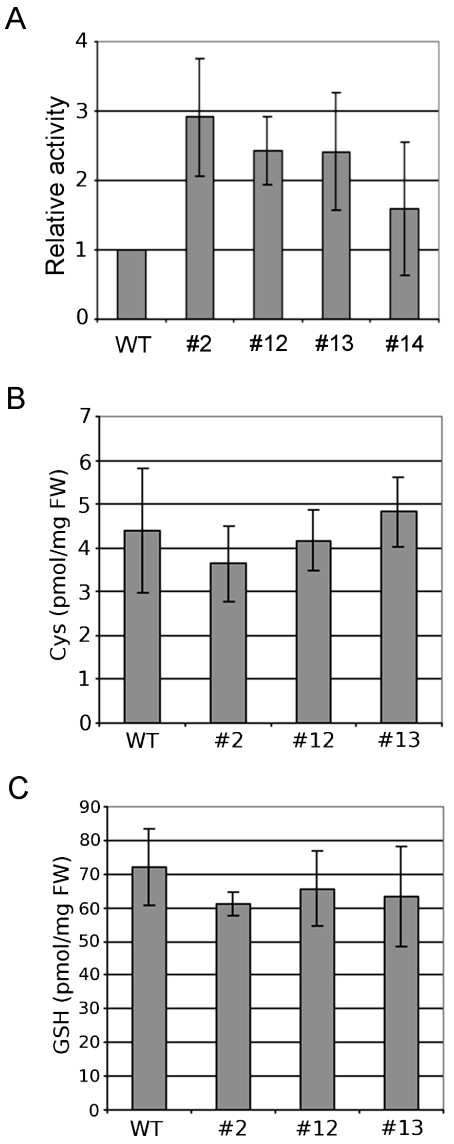

Figure 3. Properties of transgenic plants. (A) Cysteine synthase activity in leaves from the indicated plants. Crude extracts were subjected to activity assays as described in the text. Values are relative to the wild type activity set as one. All data were obtained with 3-month old T_3 plants. Each standard deviation (SD) was obtained with four independent experiments. (B, C) Estimation of cysteine and glutathione contents. Cysteine (B) and glutathione (C) were determined using youngest fully expanded leaves of 3-month old T_3 plants cultivated under non-stressed condition. After labeling with monobromobimane they were separated by HPLC as described in the text. Standard deviations (SD) were obtained with three independent experiments.

same amounts of these compounds under non-stressed conditions (Figure 3B, C). This finding is consistent with a previous report, describing that, under normal conditions, transgenic tobacco plants over-expressing spinach cysteine synthase contain the same amounts of cysteine and glutathione as non-transgenic plants (Sirko et al. 2004).

Exposure to high amounts of sodium sulfide

In order to examine the response of transgenic plants to sulfur, we exposed transgenic T_3 seedlings to sodium sulfide gas. However, our initial attempt brought out variable results, perhaps due to nutrient conditions and unstable and volatile nature of gas after prolonged treatments. Consequently, we fixed the experimental system using detached leaves, which gave stable and reproducible results under short time experimental condition. Healthy leaves were detached from control and transgenic T_3 plants, and exposed to Na_2S ($10\,mg\,l^{-1}$) in sealed bottles. Cysteine and glutathione contents were then measured by HPLC at appropriate time points (Figure 4). Within 2 h, sodium sulfide induced cysteine accumulation in all leaves up to 8-fold. The rate of increase was similar between transgenic and control plants, but the total amount was up to 2-fold higher in the former case. For example, transgenic line #13 accumulated over 80 pmole cysteine mg^{-1} fresh weight 2 h after treatment, whereas the value was 40 pmole for controls (Figure 4A). The level then gradually declined over 2 h (Figure 4A). Glutathione content was also increased up to 3-fold in leaves from lines #12 and #13 after 4 h exposure. In contrast, no obvious increase was observed in wild type leaves (Figure 4B). The 2 h difference between cysteine and glutathione in the time to reach maximal levels may indicate the time necessary for production of the latter from the former through two successive enzymatic reactions. Enhanced assimilation of sulfide to cysteine was confirmed by independent measurements after 2 h exposure to sulfide (Figure 4C, D). Transgenic lines contained 1.5-fold more cysteine than the wild type, while glutathione content was the same in both cases at this time point. This may be due to the time lag necessary for glutathione synthesis (Figure 4B). These results clearly indicated that transgenic plants accumulate more sulfur compounds in response to sulfide than wild type plants. Exposure to Na_2S for up to 72 h apparently did not induce any detectable physical damage to leaves of either wild type or transgenic plants (data not shown).

Concluding remarks

Transgenic tobacco plants over-expressing wheat cysteine synthase are reported to show higher tolerance to hydrogen sulfide and sulfite exposure than control plants (Youssefian et al. 1993, 2001). Similarly, transgenic tobacco over-expressing spinach cytosolic cysteine synthase showed a lowered sensitivity to sulfite treatment (Saito et al. 1994). The underlying mechanism appears due to a high activity of cysteine synthase, which can metabolize excess sulfide into non-toxic compounds such as cysteine and glutathione (Youssefian et al. 1993; Sirko et al. 2004). Our results are essentially consistent with this view, and further provide evidence that such

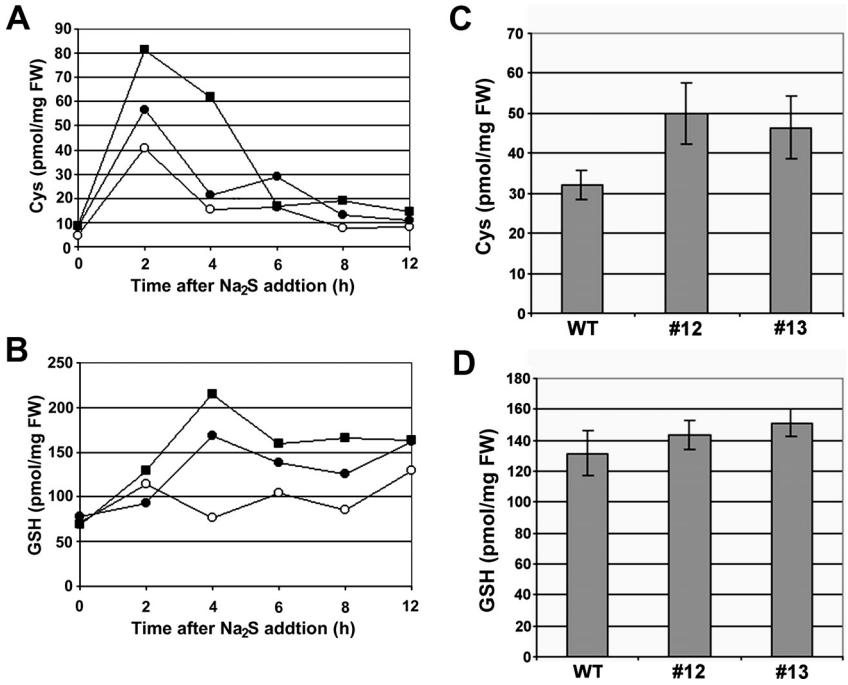

Figure 4. Effects of sulfide exposure. Accumulation of cysteine (A) and glutathione (B) after Na_2S exposure at a concentration of approximately 10 mg l^{-1} was estimated at indicated time period. Examined plants were wild type (open circle), transgenic lines #12 (closed circle) and #13 (closed rectangular). Estimation of cysteine (C) and glutathione (D) contents in the indicated plants 2 h after Na_2S exposure. Standard deviations (SD) were obtained with three independent experiments.

transgenic plants are able to efficiently assimilate toxic sulfide. Since our initial aim was to construct sulfide hyper-assimilators, rather than sulfide tolerators, the present transgenic rice might be useful given the following findings: (1) exogenous sulfide alone triggered cysteine synthase activity followed by glutathione biosynthesis, indicating precursors of cysteine and glutathione are abundant in rice; (2) rice itself has a high capacity for cysteine and glutathione biosynthesis; (3) overexpression of cysteine synthase can increase this capacity; (4) and environmental sulfide was quickly uptaken and assimilated within 2 h.

The present findings point to a feasibility of generate cysteine synthase over-expressing aquatic plants other than rice, for example, such as reeds with a large biomass for effective removal of hydrogen sulfide generated in wetlands. Our transgenic rice might themselves be practically useful in deteriorated rice fields, where hydrogen sulfide is emitted due to iron deficiency in the soil, serious damaging rice roots (National Research Council 1979).

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