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Original Paper

Hyper-assimilation of sulfate and tolerance to sulfide and cadmium in transgenic water spinach expressing an *Arabidopsis* adenosine phosphosulfate reductase

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Abstract Adenosine phosphosulfate (APS) reductase is one of key enzymes in the sulfur assimilation pathway in higher plants, catalyzing the formation of adenosine 5′-phosphosulfate from sulfate and ATP. In order to improve sulfur uptake capacity of water spinach (*Ipomea aquatica*), a plant which commonly grows wild in Southern Asia and has good potential for sequestration of environmental pollutants like sulfuric compounds, an *Arabidopsis* gene (*APR1*), encoding a plastid-resident APS reductase, was introduced into cut cotyledons via *Agrobacterium*–mediated transformation. Among 267 regenerated shoots initially obtained from 2,119 cotyledon explants, two were found to efficiently express the introduced gene and could be grown to maturity. APS reductase activity in leaves was estimated to be over 2-fold the wild-type level. Upon cultivation in the presence of 2 mM sodium sulfate, a 2.5-fold higher sulfate uptake was observed in comparison with wild-type plants. When grown in the presence of toxic levels of sulfide or cadmium, they showed a higher tolerance with increased fresh weight as compared with controls. These results suggest that transcription from the introduced gene indeed strengthened the sulfur assimilation pathway, and that the generated plants may be practically useful for phytoremediation.

Key words: Adenosine phosphosulfate reductase, *Ipomoea aquatica*, phytoremediation, sulfur assimilation.

Higher plants synthesize sulfur-containing organic compounds from sulfate, which they absorb from the environment through the sulfate assimilation pathway (Saito 2000). Sulfate is then step-wisely converted into cysteine, which serves as a principal starting metabolite for synthesis of other sulfur-containing compounds, including methionine, glutathione and secondary products (Leustek 1999).

In these pathways, adenosine phosphosulfate (APS) reductase catalyzes the initial step, converting sulfate into adenosine 5′-phosphosulfate (Bick and Leustek 1998). Its enzyme activity is considered to be regulated at both transcription and translation levels. For example, under sulfur deprivation conditions, transcript levels are markedly increased in *Arabidopsis* root tissues (Gutierrez-Marcos et al. 1996; Takahashi et al. 1997; Yamaguchi et al. 1999), and increased transcripts result in an increment in cysteine synthesis (Takahashi et al. 1997).

Heavy metal stress and nitrogen deficiency have also been found to increase transcripts and enzymatic activity of APS reductase. For example, when *Brassica juncea* was exposed to cadmium, its activity increased with a concomitant increase of cysteine and phytochelatins (Lee and Leustek 1999; Heiss et al. 1999). High levels of Cd-induced transcripts and enzyme activity were abolished by feeding cadmium-exposed *B. juncea* with cysteine or glutathione (Lee and Leustek 1999; Heiss et al. 1999). Furthermore, APS reductase activity is closely related with nitrogen status. When *Arabidopsis* was cultured without nitrogen source for 72 h, the activity decreased to 70% and 50% of the control values in leaves and roots, respectively (Yamaguchi et al. 1999; Koprivova et al. 2000). Northern and Western analysis confirmed correlations between APS reductase activity and both transcript and protein levels (Koprivova et al. 2000). Such reduction was fully reversed within 24 h of addition of nitrogen sources including NO_3^-, NH_4^+ and glutamine (Koprivova et al. 2000). *O*-Acetylserine (OAS) was subsequently found to be the rate-limiting factor,

Abbreviations: APS; Adenosine phosphosulfate: MS; Murashige-Skoog.

requiring nitrogen for its synthesis, and regulating the whole sulfur assimilation pathway in a feed-back mode (Yamaguchi et al. 1999; Koprivova et al. 2000).

Water spinach (*Ipomoea aquatica*) is a common aquatic plant in Southeast Asia, vigorously growing even in rather polluted wetlands, such as the Mae Moh mine drainage area contaminated with high concentration of sulfate (800–2,000 mg/l) in Thailand. However, whether efficient accumulation or exclusion sulfuric compounds are the bases of resistance have yet to be clarified in detail. Our long-range aim is to use this plant to remove pollutants by conferring an elevated potential for sulfate assimilation. For this purpose, we introduced an *Arabidopsis* APS reductase gene (*APR1*) into *I. aquatica* and evaluated the response of transgenic plants to pollutants. Here we describe that they indeed show a high capacity for sulfate uptake and also tolerance to toxic levels of sulfide and cadmium.

Materials and methods

Plant materials, culture media and chemical treatments

Seeds of water spinach (*I. aquatica*) were purchased from Chia Tai Co. Ltd. (Thailand), and used for further experiments. For biochemical assays, they were sown on soil and grown in a growth cabinet to maturity. For tissue culture, surface sterilized seeds were plated on modified MS solid medium (Murashige and Skoog 1962), containing 1/2-strength MS inorganic salts supplemented with 100 mg/l inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l folic acid, 30 g/l sucrose and 2.5 g/l gellan gum (Mori et al. 1999). This was used as the basal medium. Germinated seedlings were then cultured at 25°C under a 16 h/8 h photoperiod at a light intensity of 3,000 luxs (white fluorescent tube). For stress treatments, shoots were cultured in MS medium containing 0.5 mM sodium sulfide or 50 μM cadmium chloride, cultivated for 1 month, and fresh weights were measured.

Construction of transformation vectors

The Arabidopsis gene encoding a chloroplastid isoform of APS reductase was amplified by PCR using a cDNA library of *Arabidopsis* (Columbia) as the template. The forward and reverse primers were 5′-C-TGTGAAGATGGCAATGTCTGTAAATG-3′ and 5′-G-GATCACAAAGTCAGAGATAAGCTCTGCT-3′, to respectively. give *Xba*I and *Xho*I sites at the 5′ terminus. The PCR product of 1,558 bp proved 100% homologous to *APR1* (*A. thaliana* 5′-adenylsulfate reductase, accession number AFO016282). After digestion with *Xba*I and *Xho*I, cDNA was ligated into the pBIH1-IG(SX) plasmid modified from pBIH1-IG (Kimura et al. 1993), amplified in *E coli* DHα and finally used for *Agrobacterium tumefaciens* EHA101 (Heiss et al. 1999) transformation (Khamwan et al. 2003).

Plant transformation

Cotyledon segments containing petiole-like cotyledon bases (Akaracharanya et al. 2001) were excised from one week-old seedings and used as explant sources. Tranformation was performed as described with modifications (Khamwan et al. 2003). Briefly, 200 cotyledon segments were suspended in 20 ml of MS medium containing 9.0×10^6 cells/ml *A. tumefaciens* and 50 μM acetosyringone and incubated at 25°C for 3 days in the dark. After washing with 300 mg/l cefotaxime, materials were cultured on modified MS solid medium containing 10 μM thidiazuron and 300 mg/l cefotaxime at 25°C under a 16 h/8 h photo cycle for 1 month. On reaching 2 cm in height, regenerated shoots were transferred to a culture box containing modified MS solid medium with 10 μM thidiazuron, 25 mg/l hygromycin and 300 mg/l cefotaxime and incubated for 1 month. Regenerated shoots which survived after 1 month were transferred to hormone-free MS solid medium containing 300 mg/l cefotaxime and incubated further under light. Plantlets with well developed shoots and roots were transferred to 1/4-strength MS solid medium without supplement, acclimated for 1 week and finally transferred to glass house conditions.

Southern hybridization

DNA was extracted from leaves as described (Khamwan et al. 2003) and 10 μg aliquots were double-digested with *Xba*I and *Xho*I, and subjected to Southern hybridization using an *APR1* fragment isolated from pBIH1/*APR1* by *Xba*I-*Xho*I digestion as a probe. Labeling and detection were performed using a DIG High Prime kit (Boehringer Manheim GmbH, Germany).

APS reductase assay

APS reductase activity was quantified by the method described by Kano et al. (1993). All procedures for extraction and fractionation were performed at 4°C. A sample of 4 g (wet weight) of water spinach (*I. aquatica*) leaves was ground in an ice-cold motar with liquid nitrogen and 6 ml of 0.2 M phosphate buffer, pH 8.0, containing 10 mM EDTA and 1 mM 2-mercaptoethanol. After filtration through cheesecloth and centrifugation at $10,000\times g$ for 20 min, the supernatant was used as a crude enzyme solution. APS reductase activity was determined by measuring thiol-dependent conversion of APS to AMP. A 500 μl reaction mixture contained 25 μl 1 mM glycine-NaOH buffer pH 9.5, 5 μl 10 μM APS, 5 μl 1 mM dithiothreitol, 125 μl Na_2SO_4 and 340 μl crude enzyme solution. After preincubation for 2 min at 25°C, the reaction was started by addition of crude enzyme solution, and after 5 min was terminated by addition of 100 μl of 1 M acetic acid. This addition

brought the pH of the incubation mixture to about 5.0 to 5.5, at which APS reductase exhibits no activity, and no spontaneous hydrolysis of APS occurs. A control reaction without DTT was run for each assay to correct for thiol-independent hydrolysis of APS. The incubation mixture was then filtered through a Millipore ultrafree C3GV filter (0.22 μm) (Millipore Corporation; Bedford USA), and fractionated with an HPLC 1100^{TM} (Agilent Technologies) on a TSK $ODS_{80}T_M$ column (4.0 mm× 250 cm). A 5-μl sample was injected and separated under isocratic conditions using binary pumps : pump A, 0.1 M KH_2PO_4 containing 10 mM tetra-n-butyl-ammonium hydrogen sulfate, pH 5.8 (adjusted with 1 N NaOH) ; pump B, acetonitrile at flow rate 0.8 ml/mm. One unit of APS reductase was defined as the amount of enzyme that liberated 1 nmol of AMP in 1 min at pH 9.5 at 25°C. Triplicate measurements were conducted for each sample.

Measurement of sulfur compounds

For sulfate estimation, 1 month-old plantlets were oven dried at 80°C for 48 h and sulfate in the residues was analyzed by a standard turbidimetric method (American Public Health Association 1992). Briefly, samples were suspended in 3% $MgCl_2 \cdot 6H_2O$, 0.5% sodium acetate, 0.1% potassium nitrate, 2% acetic acid, mixed with 0.75 g of $BaCl_2$ (20–30 mesh) crystal, stirred for 60±2 sec at constant speed, and measured at A_{420} for barium sulfate turbidity. Sulfate concentrations were determinedby referring to a standard curve with known concentrations. Triplicate experiments were performed for each condition. Cysteine and glutathione were quantified by the method described by Noctor and Foyer (1998). Sample leaves (100 mg) were frozen in liquid nitrogen and ground in 600 μl of 0.1 N HCl containing 1 mM EDTA solution. After centrifugation at 10,000×*g* at 4°C for 30 min, the supernatant was labeled with monobromobimane, and a 100 μl aliquot was mixed with 20 μl of 10 mM DTT, adjusted pH to 8.0 and immediately mixed with 20 μl of 3 mM monobromobimane in acetonitrile. The mixture was kept in the dark at room temperature for 15 min, then mixed with 0.6 ml of 10% acetic acid and kept on ice for 5 min. Supernatant obtained from centrifugation at 5,000×*g* at 4°C for 2 min was analyzed by HPLC (HP1100, Hewlett Packard, USA) with a Hypersil ODS 5 μm column (4 mm× 125 mm) (LiChrospher-100, Hewlett Packard, USA) and a fluorometer fitted with an OPA emission filter detector (excitation at 384 nm and emission at 462 nm) (Hewlett Packard, USA). The sample was eluted at 1 ml/min flow rate by a gradient of mobile phase: (A) 10% methanol, 0.25% acetic acid, pH 4.3 and (B) 90% methanol, 0.25% acetic acid, pH 4.3. Retention times of cysteine and glutathione were 3.55 and 4.01 min, respectively. Triplicate measurements were performed for each sample.

Results and discussion

Generation of transgenic water spinach

Water spinach (*I. aquatica*) was transformed with *APS1* as described in our previous report (Khamwan et al. 2003). Among 2119 cotyledon segments which were initially infected with *A. tumefaciens* harbouring pBH1/APR1, 267 regenerated shoots. However, only two lines (#2 and #8) were found to be resistant to 25 μg/ml hygromycin. These were cultivated to maturity in a glass house and used for further studies. The phenotype in both cases was the same as for wild type control plants, with normal growth and features (Figure 1A). The presence of the introduced gene could be confirmed by Southern hybridization, showing a clear single signal at the 1560 bp position in both lines #2 and 8 (Figure 1B). Since the size of fragment was the same as that of the introduced gene, and since the control untransformed plant did not contain any sequence hybridizing to the probe (Figure 1B), we concluded that both lines had successfully incorporated a single copy of the *APS1* gene. Expression of the introduced gene was examined by assaying enzymatic activity of the encoded protein. When crude protein extracts were subjected to APS reductase reactions, the transgenic lines exhibited 2- to 2.5-fold higher activity than the controls (Figure 1C). These results indicated transgenic water spinach plants to express intact *Arabidopsis* APS reductase having full activity.

Assimilation of sulfur compounds

Transgenic plants were assayed for their assimilation of sulfur compounds. When cultivated in the presence of 2 mM sulfate for 1 month, transgenic lines #2 and #8 absorbed 185 and 178 mg sulfate/g dry weight, respectively, in contrast to only 71 mg by the controls, a 2.5-fold increase (Figure 2A). Phenotype and growth rate were essentially the same in the two lines (data not shown). The results indicated that the introduced gene functionally strengthened the sulfur assimilation pathway. Since the initial stable product in the sulfur assimilation pathway is cysteine, and since the major sulfur compound derived from this is glutathione, we subsequently estimated their amounts in transgenic and wild type plants grown under standard cultivation conditions. In wild type plants, cysteine was present at approximately 2 nmol/g fresh weight of young leaves. In transgenic lines #2 and #8, the values were 2.6 and 4.7 nmol/g fresh weight, respectively (Figure 2B). Glutathione content was 29 nmol/g fresh weight in the controls, and 25 and 78 nmol/g fresh weight in transgenic lines #2 and #8, respectively (Figure 2C). Although the glutathione level in line #2 was similar to the control value, these results indicate a clear tendency toward increase of sulfur assimilation capacity in transgenic

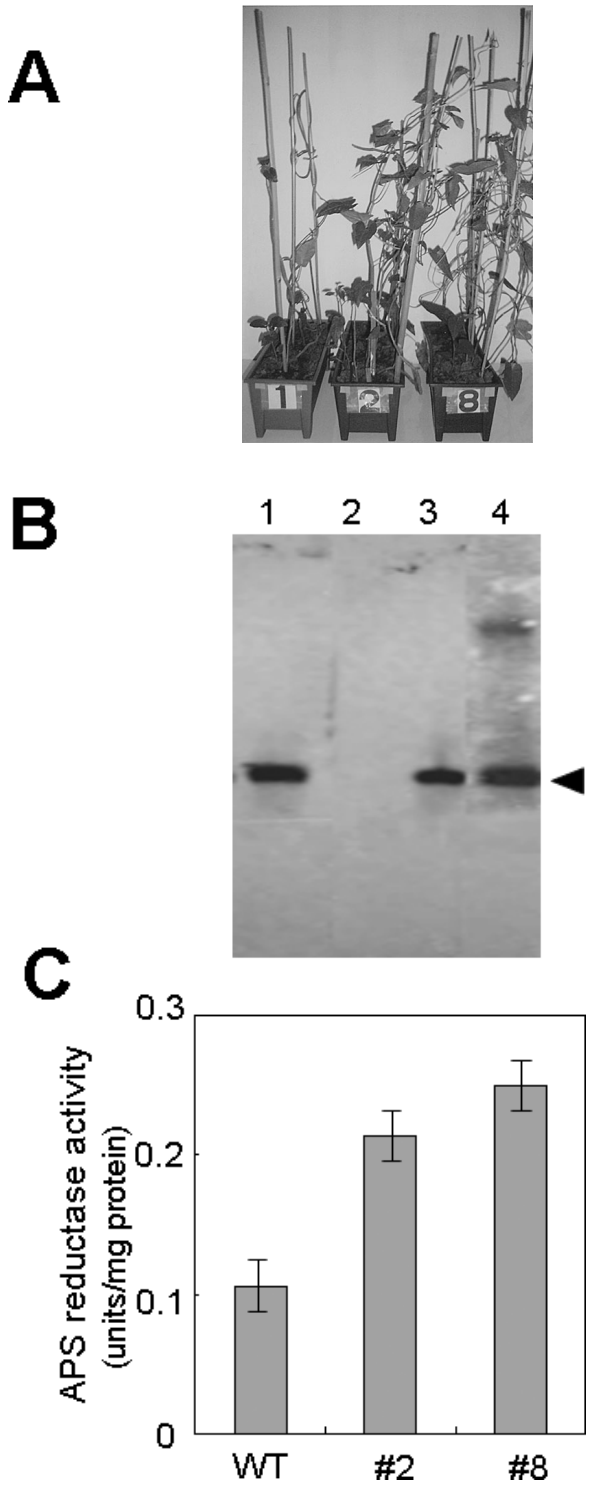

Figure 1. Properties of transgenic water spinach. (A) Phenotype of mature plants; wild type (left), transgenic line #2 (middle) and #8 (right). (B) DNA blot hybridization. A 10-μg aliquot of DNA was digested with *Xba*I and *Xho*I, fractionated on a 0.8% agarose gel, transferred onto a nylon membrane, and subjected to hybridization with the *APS* probe. Samples are the plasmid containing *APS* (lane 1), and DNA from wild-type water spinach (lane 2), or transgenic lines #2 (lane 3) and #8 (lane 4). The arrowhead indicates the position of a 1.6 kb DNA fragment. (C) Enzymatic activity of APR. Crude extracts were prepared from wild type (WT), and transgenic lines #2 and #8, and subjected to assays as described in the text. Standard deviations were calculated from triplicate measurements.

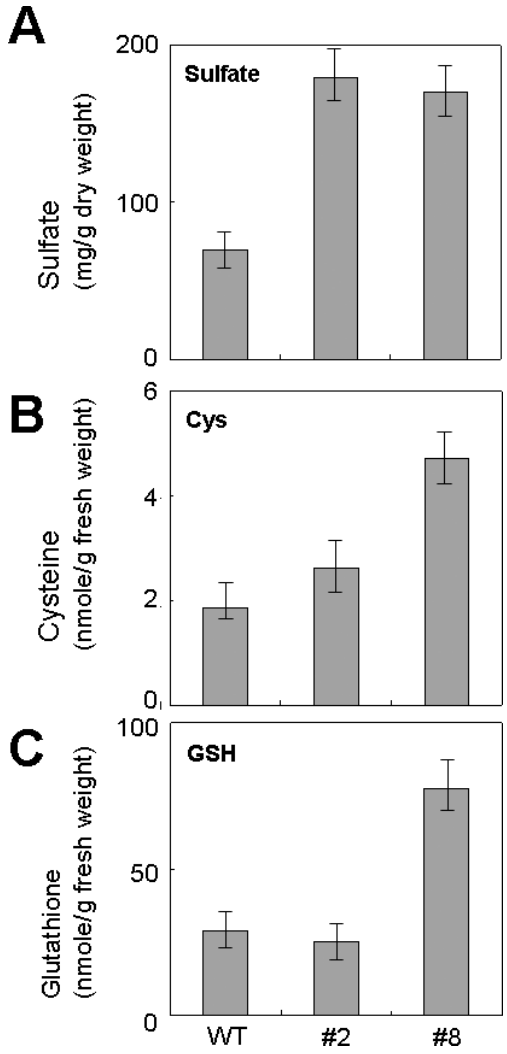

Figure 2. Amounts of sulfur-containing compounds. Fresh materials were processed to estimate sulfate (A), cysteine (B) and glutathione (C) levels by HPLC as described in the text. The amount of sulfate is expressed on a dry weight basis, and values for cysteine and glutathione are on a fresh weight basis. Measurements were made in triplicate and standard deviations are shown by bars. GSH is glutathione.

plants.

Sulfide and cadmium tolerance

Stress responses of transgenic plants were then examined. Both wild type and transgenic lines were grown for 1 month in the presence of 0.5 mM sodium sulfide or 50 μM cadmium chloride, and their phenotypes and fresh weights were assessed. The control wild type plants showed severe symptoms of toxicity with the two treatments, becoming yellow and ultimately dying. Fresh weight increased only fractionally in the presence of sulfide (Figure 3A) and not at all with cadmium (Figure 3B). Transgenic plants also exhibited symptoms, but suffered much less in comparison with the controls, demonstrating 3 to 5-fold greater increase in fresh weight in the presence of sulfide (Figure 3A).

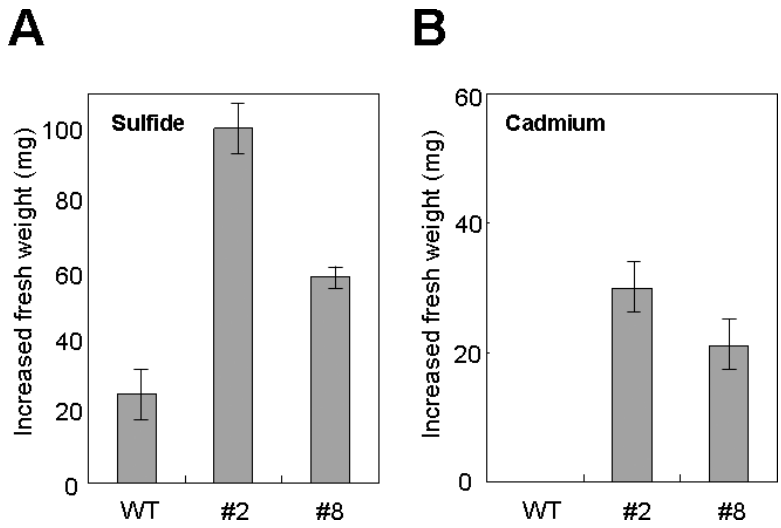

Figure 3. Stress tolerance of transgenic water spinach. Healthy plantlets were cultivated for 1 month in the presence of 0.5 mM sodium sulfide (A) or 50 μM cadmium chloride (B). The average initial fresh weights of three samples for sulfide experiments were 150 mg for the wild type (WT), and 60 mg and 190 mg for lines #2 and #8, respectively. Those for cadmium experiments were 150 mg for WT, 190 mg for #2 and 120 mg for #8.

In the presence of cadmium, they also grew, exhibiting increased fresh weight of approximately 30 and 20 mg in lines #2 and #8, respectively (Figure 3B). They did not die at least within the observation period of two months, although growth rate and other features were affected. Thus, it was concluded that the introduced APS reductase gene conferred an elevated resistance to sodium sulfide and cadmium on the water spinach.

Concluding remarks

Because of its capacity to grow in polluted water, water spinach has been proposed to have potential utility for purification purposes (Furukawa and Fujita 1993). In addition, it accumulates significant amounts of toxic metals such as Fe, Cu, Cr, Mn and Pb in its leaves (Rai and Sinha 2001). It requires only two weeks of cultivation at an appropriate temperature after sowing and after decapitation of main stems, new shoots form from each stem nodule, making continuous harvesting possible. Considering these properties, we previously suggested practical application of this plant for phytoremediation after strengthening the sulfur uptake capacity by genetic modification (Akaracharanya et al. 2001; Khamwan et al. 2003). In the present study, we successfully generated transgenic water spinach, which absorbed 2.5-fold more sulfate from their environment than the wild type, and demonstrated resistance to toxic levels of sulfide and heavy metals. Although the mechanisms by which absorbed sulfur and heavy metals are detoxified and sequestered must be determined, our transgenic plants appear to be useful for mass cultivation, offering the possibility of detoxifying large areas of polluted wetland.

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