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Original Paper

# Simultaneous expression of serine acetyltransferase and cysteine synthase results in enhanced sulfate uptake and increased biomass in *Ipomaea aquatica*

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**Abstract** The long range goal of the present study is to practically utilize *Ipomoea aquatica* (water spinach) for phytoremediation of polluted water with sulfuric compounds. In higher plants, the sulfate assimilation pathway consists of 5 key enzymes, among which serine acetyltransferase (SAT) and cysteine synthase (CS) constitute one of the rate limiting steps. Subsequently we have attempted to improve the sulfur assimilation capacity of *I. aquatica* using genes encoding these two enzymes. Cotyledon segments of seedlings were transformed with *Arabidopsis SAT* and rice *CS* genes under the control of the cauliflower mosaic virus 35*S* promoter. Among 3,245 cotyledon explants, 325 regenerated shoots, and two showed a high tolerance to hygromycin, designated as SR3 and SR10. In transgenic lines, the SAT activity was over 2-fold, and the CS was 3-fold higher than those in the wild type control. The cysteine and glutathione contents were also 6- and 2-fold higher than the control, respectively. When cultured in the presence of 1 g $l^{-1}$ (7 mM) sulfate, they accumulated sulfate as much as 20 mg $g^{-1}$ fresh weight, being 5-fold higher than the control. Under standard culture conditions, transgenic lines grew faster than the control, showing a 20% increase in fresh weight within 5 weeks cultivation. These results suggested that strengthening of SAT and CS resulted in increase not only in sulfate uptake, but also in total biomass.

**Key words:** Cysteine synthase, *Ipomoea aquatica*, phytoremediation, serine acetyltransferase, sulfate.

Lignite coal is one of the main fuels for generating electricity in Thailand. Waste water generated from mining process in the Mae Moh lignite mine was found to contain a high concentration of sulfate ion (800–2,000 ppm), which is highly detrimental for the surrounding environment. In order to remove these sulfate ions, the wet land method was introduced, in which water is siphoned through an anaerobic pond, where sulfate ion is reduced to hydrogen sulfide by anaerobic bacteria. Hydrogen sulfide is then removed by ventilation. However, only 30% of sulfate was found to be removed by this method, leaving water to retain still a too high concentration of sulfate to be released to natural water resources. Consequently, water is again pooled in a large reservoir (biological pond) to dilute sulfate by mixing with nearby natural water.

Plants absorb sulfate from the environment through the sulfate assimilation pathway, which is constituted of 5 major enzymes (Saito 1999, 2000) (Figure 1A). The incorporated sulfate is first converted into adenosine 5′-phosphosulfate (APS) catalyzed by ATP sulfurylase. APS is then reduced to sulfite by APS reductase, and sulfite is again reduced to sulfide by sulfite reductase. The resulting sulfide is incorporated into cysteine by cysteine synthase (*O*-acetyl-L-serine[thiol]lyase or CS), which uses *O*-acetyl-L-serine (OAS) as the sulfide acceptor. The OAS is synthesized from serine and acetyl CoA by serine acetyltransferase (SAT) (EC 2.3.1.30). The whole steps are well regulated, for example, by a feed-back system that prevents overproduction of toxic intermediates (Saito 2000; Noji et al. 2001), but the rate-limiting step is considered to be the first and the last steps, i.e., APS and cysteine syntheses.

During the past years, we have intensively examined the possibility of utilizing *I. aquatica* (water spinach or pakbung), a fast growing aquatic plant, for the remediation of sulfur-polluted water as described above by strengthening its sulfur assimilation pathway by the

Abbereviations: APS, adenosine 5′-phosphosulfate; CS, cysteine synthase; OAS, *O*-acetyl-L-serine; SAT, serine acetyltransferase.
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genetic engineering technology. To this end, we have established the regeneration and genetic transformation systems of this plant (Akaracharanya et al. 2001; Khamwan et al. 2003). Recently, we have successfully constructed a transgenic *I. aquatica* expressing APS reductase, which showed a high assimilation capacity of sulfate (Sakulkoo et al. 2005). In the present study, we transformed *I. aquatica* with a dual-gene vector containing *CS* and *SAT*, the latter particularly playing a critical role in the pathway by directly supplying OAS, and we showed that the resulting transgenic plants accumulated a high level of sulfur compounds with a fast-growing phenotype.

## Materials and methods

### Plant materials and culture condition

Seeds of water spinach (*I. aquatica*) were purchased from Chia Tai Co. Ltd. (Thailand), and used throughout experiments as previously described (Sakulkoo et al. 2005). Briefly, surface sterilized seeds were plated on MS solid medium (Murashige and Skoog, 1962), and germinated seedlings were then cultured in a growth cabinet at 25°C under a 16 h/8 h photoperiod. For sulfate treatments, shoots were cultured in modified MS medium containing 1000 mg $l^{-1}$ (7 mM) sodium sulfate, cultivated for 1 month, and fresh weights were measured. Biomass estimation was assessed by periodically measuring fresh weight of hydroponically cultured plantlets using Hoagland nutrient solution in a greenhouse.

### Construction of transformation vectors

The cytosolic *CS* gene of rice (*rcs1*, Nakamura et al. 1999) was amplified by PCR and inserted into a plasmid pGIH1-IG(SX) at *Xba*I and *Sac*I sites (pBIH1-IG(SX)-rcs1). The cDNA of Arabidopsis *SAT1* (EST clone 104E8T7) was obtained from Arabidopsis Biological Resource Center (The Ohio State University) and cloned into pGEM-7Z (Promega) at *Sma*I and *Bam*HI sites (pGEM-*SAT1*). After double digestion of pGEM-*SAT1* by *Xba*I and *Sac*I, *SAT1* was replaced with *rc1* of pBIH1-IG(SX)-*rcs1* at *Xba*I and *Sac*I sites (pBIH1-IG(SX)-*SAT1*). The pBIH1-IG(SX)-*rcs1* was double digested with *Xba*I and *Bam*HI to isolate the *rcs1-HPT* fragment, which was temporarily cloned in pUC19. The *rcs1-HPT* fragment was isolated by *Sal*I and *Bam*HI digestion and ligated to plasmid pGIH1-IG(SX)-*SAT1* at *Sal*I and *Bam*HI sites (pBIH1-IG(SX)-*SAT1-rcs1*) (Figure1B).

### Plant transformation

Transformation was essentially performed as described (Sakulkoo et al. 2005). *Agrobacterium tumefaciens* EHA 101 (Hiei et al. 1994) harbouring plasmid pBIH1-IG(SX)-*SAT1-rcs1* was used throughout the experiments. The regenerated shoots survived after one month on modified MS solid medium (1/2-strength MS inorganic salts supplemented with 100 mg $l^{-1}$ inositol, 0.1 mg $l^{-1}$ thiamine-HCl, 0.5 mg $l^{-1}$ folic acid, 30 g $l^{-1}$ sucrose, 2.5 g $l^{-1}$ gellan gum) containing 25 mg $l^{-1}$ hygromycin and 300 mg $l^{-1}$ cefotaxime were transferred to hormone-free MS solid medium containing 300 mg $l^{-1}$ cefotaxime and incubated at the same condition under continuous light. Plantlets with well developed shoot and root were selected and grown in Hoagland nutrient solution in a controlled-environment greenhouse.

### RT-PCR

Total RNA was isolated from the first young fully expanded leaf of transgenic and wild type plantlets using SV Total RNA Isolation System (Promega) according to the manufacturer protocol. Five hundred nanograms of total RNA were used for reverse transcription using RNA PCR kit (AMV) ver. 3.0, (Takara, Kyoto). A-1 $\mu$l aliquot of the RT products was used for nested PCR for detection of *SAT1* and *rcs1* transcripts. Two sets of primers were used for *rcs1*; forward 1, 5′-ATAATGGGT-GAGACCATCGC-3′ (cDNA position 71–90), forward 2,5′-GCAGCGAAGACAAACAACTC-3′ (cDNA position 479–498); reverse, 5′-TCTGAGCGAGCCTAACAGCT-3′ (cDNA position 901–920). Similarly two sets for *SAT1* were prepared; forward 1,5′-CTACGCTTCGATC-ACATCTCA-3′ (cDNA position 219-239), reverse 1,5′-ATCACATAATCAGACCACTCGG-3′ (cDNA position 950–928), forward 2, 5′-GGGCTTCAAAGGCTTCCT-CGC-3′ (cDNA position 435–455), reverse 2,5′-GGA-ATCTTATCATGTTTTCTCGG-3′ (cDNA position 896–873). As the internal control, ubiquitin cDNA was used with forward primer, 5′-GGATGAACGCTGGCGGCAT-GC-3′ (position 13–33), and reverse primer, 5′-TAGAC-AAAGCACATCACGACC-3′ (position 456–476). The PCR was performed by a 25-cycle of 94°C 30 sec, 55°C (or 50°C) 30 sec, 72°C 1 min, and a final extension at 72°C 7 min (GeneAmp 2400, Perkin Elmer) and the products were fractionated on 0.9% agarose gel.

### Enzyme activity assay

Cysteine synthase activity was estimated as described (Youssefian et al. 1993). Using crude extracts containing 1 $\mu$g protein, cysteine was synthesized in a 100 $\mu$l reaction mixture containing sodium sulfide and *O*-acetyl L-serine as substrates and 5′-pyridoxal as a co-factor, and the amounts produced were estimated by the method described by Gaitonde (1967). SAT activity was determined as described (Baecker and Wedding 1980; Noji et al. 2001). Protein concentration was quantified by the Bradford method (Bradford 1996).

### Cysteine and glutathione contents

The amounts of cysteine and glutathione were quantified by HPLC (Noctor and Foyer 1998) using a 100-mg leaf

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tissue from transgenic and wild type plants as previously described (Sakulkoo et al. 2005).

### *Sulfate uptake*

Transgenic and wild type plantlets were cultivated in 25 ml of modified MS medium containing 1,000 mg $l^{-1}$ sulfate and 300 mg $l^{-1}$ cefotaxime for 2 weeks. Tissues were then washed with distilled water, blotted dry and weighed. The amounts of sulfate residue in modified MS medium were analyzed by HPLC (ED50, Dionex) equiped with an IonPac AS11 column (4×250 mm) and a conductivity detector. The sample was eluted by 12 mM potassium hydroxide at a flow rate of 1 ml $min^{-1}$. Retention time of sulfate was 3.00 min. Triplicate measurements were conducted for each sample.

## Results and discussions

### *Simultaneous expression of SAT and CS*

In order to strengthen the sulfur assimilation, both *CS* and *SAT* genes were simultaneously introduced into *I. aquatica* using a multigene transformation vector under the control of cauliflower mosaic virus (CaMV) 35*S* promoter (Figure 1B). Among 3,245 cotyledon segments, which were initially infected with *Agrobacterium tumefaciens* harbouring plasmid pBIHl-IG(SX)-*SAT1-rcs1*, 325 regenerated shoots. Subsequent selection by 25 $\mu$g $ml^{-1}$ hygromycin finally yielded only two survivals, which efficiently expressed both *SAT1* and *CS1* as confirmed by RT-PCR analysis (Figure 1C). They were designated as SR3 and SR10, and cultivated to maturity in a controlled-environment condition for further study. The enzymatic activities were estimated by *in vitro* assay using crude extracts from mature leaves grown under unstressed condition. Both transgenic SR3 and SR10 lines showed elevated levels of SAT activity at 2.5- and 2-fold that of the wild type control, respectively (Figure 2A). The CS activity was also increased, each showing 4- and 3.4-fold higher than the control, respectively (Figure 2B). It was concluded that the obtained transgenic lines simultaneously and constitutively expressed functional enzymes from the transgenes.

### *Assimilation of sulfur compounds*

The initial product in the sulfur assimilation pathway is cysteine, and the major sulfur compound derived from this is glutathione. Since the transgenic lines expressed elevated activities of cysteine synthetic enzymes, we estimated their amounts in transgenic and wild type plants grown under standard cultivation conditions. In wild type plants, cysteine was present at approximately 8 nmol $g^{-1}$ fresh weight of young leaves. In transgenic lines SR3 and SR10, the values were 63 and 54 nmol $g^{-1}$ fresh weight, being 8- and 7-fold higher than the

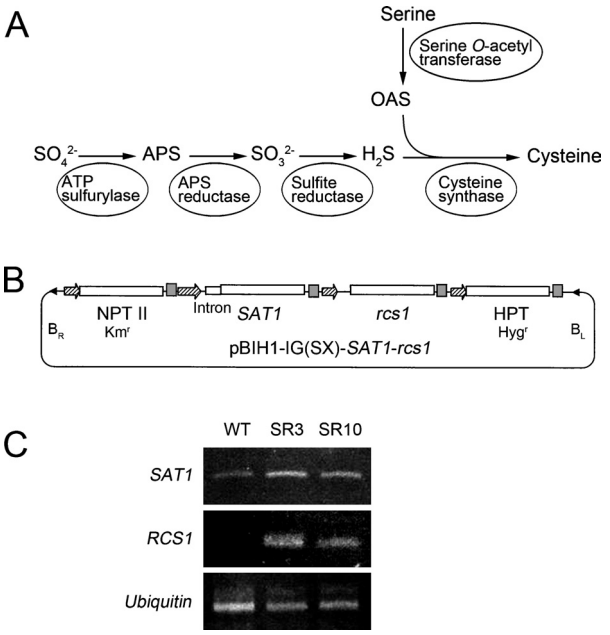


Figure 1. Construction of transgenic *I. aquatica*. (A) Sulfur assimilation pathway in higher plants. (B) Schematic illustration of pBIH1-IG(SX)-*SAT1-rcs1* used in transformation. CaMV 35S promoter and Nos terminator are indicated by hatched arrow and grey square, respectively. (C) RT-PCR analyses of transgene expression. Transcripts for *SAT1* and *rcs1* were detected by the nested-PCR using two sets of primers as described in the text. As the internal control, ubiquitin transcripts were analyzed.

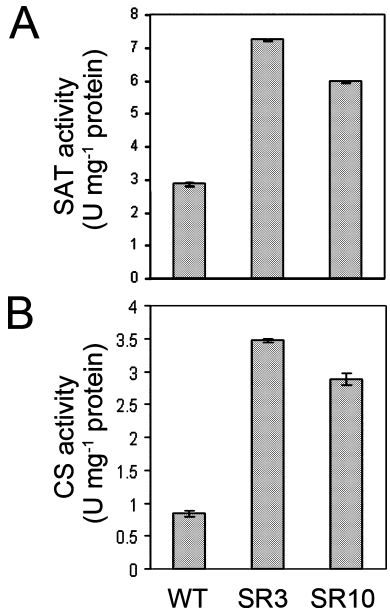


Figure 2. Enzymatic activities in transgenic plants. Crude extracts were prepared from wild type (WT), and transgenic lines SR3 and SR10, and subjected to assays as described in the text for SAT activity (A) or CS activity (B). Standard deviations were calculated from triplicate measurements.

wild type, respectively (Figure 3A). Glutathione content was 17 nmol $g^{-1}$ fresh weight in the control, while 23 nmol $g^{-1}$ fresh weight in the SR3, and 52 nmol $g^{-1}$ fresh weight in the SR10 (Figure 3B). Each ratio to the

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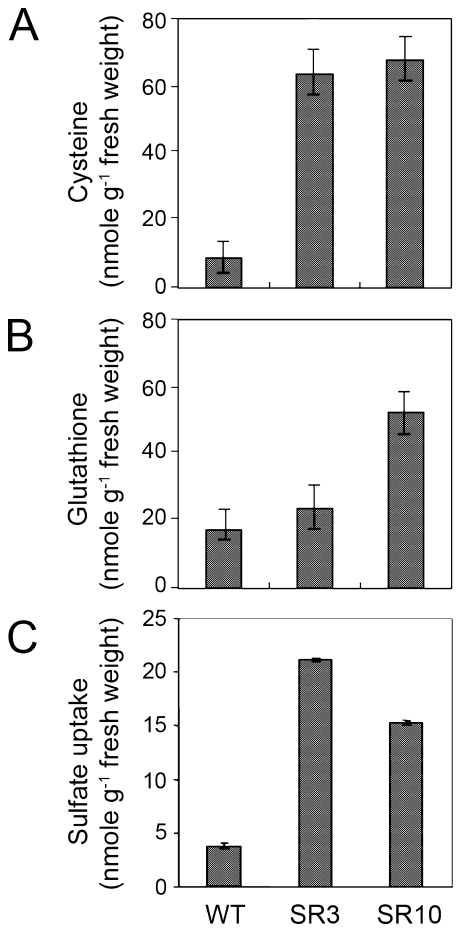


Figure 3. Amounts of sulfur-containing compounds. Fresh materials were processed to estimate cysteine (A), glutathione (B) and sulfate (C) levels by HPLC as described in the text. Sulfate uptake was measured after cultivation of plantlets in the presence of 1000 mg $l^{-1}$ sodium sulfate. The amount is expressed on a fresh weight basis. Measurements were made in triplicate and standard deviations are shown by bars. GSH is glutathione.

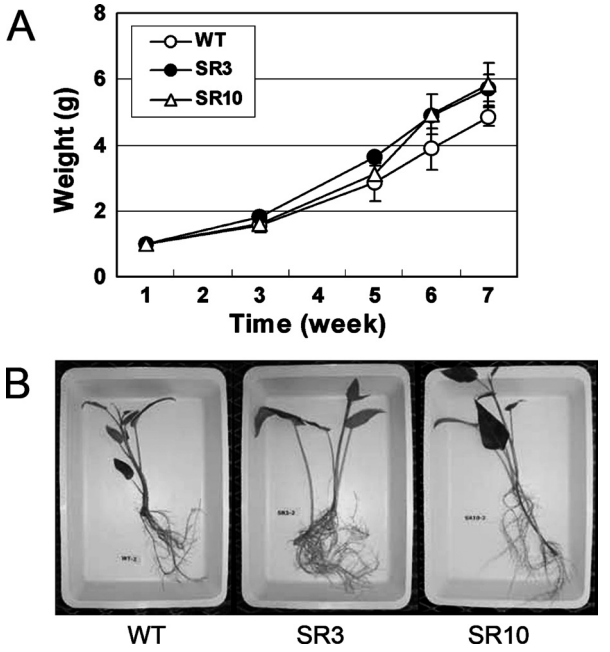


Figure 4. Fast growth of transgenic plants. (A) Time course of growth. Seedlings were prepared from cuttings of the indicated transgenic line and wild type plants, hydroponically cultured in Hoagland nutrient solution, and the total weight was measured at indicated time point. Values are the average from 5 independent samples of each line. (B) Plant features after 5-week cultivation. Typical samples from the indicated line and wild type plants were photographed 5 weeks after culture initiation.

control was 1.4- and 3-fold, respectively. Although the glutathione level in line SR3 was similar to the control value, perhaps due to quick metabolism, these results indicate a clear tendency toward increase of sulfur assimilation capacity in transgenic plants. In order to assess whether observed increase in sulfur assimilation capacity contributes to stress tolerance, transgenic lines were hydroponically cultivated in modified MS medium containing 1,000 mg $l^{-1}$ (7 mM) sulfate for 2 weeks, and then the amount of sulfate uptaken was analyzed. Results showed that the wild type, SR3 and SR10 plants absorbed approximately 4, 21 and 15 mg sulfate $g^{-1}$ fresh weight, respectively (Figure 3C).

### *Increase of biomass*

During the initial cultivation, we realized that both transgenic lines grew faster than the control, showing increased size in leaves and plant heights (data not shown). Subsequently, five plantlets from wild type and each transgenic lines were hydroponically cultivated in a greenhouse and the growth rate and weight were periodically measured (Figure 4). Transgenic lines showed a fast growth, which was evident after 5-week culture, and after 7 weeks, the weight was more than 20% heavier than the control (Figure 4A). Phenotypically, they also exhibited larger leaves and longer stems than the control (Figure 4B). Since the culture condition was the same for all plants examined, the observed increase in biomass was apparently the results of increased sulfur uptake by transgene expression.

### *Concluding remarks*

In the present study, we established the method of multigene transformation into *I. aquatica*. Upon simultaneous expression of CS and SAT, plants showed a marked increase not only in sulfate uptake but also in biomass. The former was along the expected line, and plants are potentially applicable for practical use for absorption of sulfur-polluted fresh water (see introduction). The latter was unexpected, and its underlying mechanism is currently not clear. It is conceivable that efficient sulfur supply might have enhanced carbon and nitrogen assimilation, all being conducted in chloroplast under well-controlled network system (Dennis and Blakeley 2000). If it is the case, the present finding may provides a novel idea for

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molecular breeding, in which reinforcement of multiple pathways for nutrient assimilation should be intensively undertaken. Indeed, the rate of carbon assimilation was shown to be increased by expressing a transgene for fructose bisphosphatase (Yokota and Shigeoka 2006). We also previously generated transgenic *I. aquatica*, expressing adenosine phosphosulfate reductase, which catalyzes the first step of sulfur assimilation (Figure 1A). The resulting plants exhibited not only an increased uptake of sulfate but also a tolerance to toxic levels of sulfide and a heavy metal, cadmium (Sakulkoo et al. 2005). Taken these observations together with the present finding, it is practically possible to construct transgenic plants possessing multiple genes involved in nutrient assimilation pathways by crossing or by multigene transformation, thereby contributing not only to phytoremediation, but also to increasing biomass, which is an important trait for materials production.

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