Sulfate Phytoremediation through Transgenic *Ipomoea aquatica* Forsk. Harbouring Rice (*Oryza sativa* L.) Cysteine Synthase Gene

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Rice (*Oryza sativa* L. cv Nipponbare) cysteine synthase gene (*rcs*1) encoding cytosolic isoform was transformed into *Ipomoea aquatica* Forsk. using *Agrobacterium tumefaciens* EHA101 harbouring plasmid pBIH1-IG(SX)-RCS1. Among 240 regenerated shoots obtained from 908 cotyledon explants, 2 shoots designated as IRCS2 and IRCS4 were tolerant to 25 μg/ml hygromycin and their genomic DNAs gave the expected *rcs*1-PCR band when used as a template with specific oligonucleotide primers for amplifying *rcs*1. Cysteine synthase activity of the transformants, IRCS2 and IRCS4, was 3.4 and 3.0 times higher than those of wild type, respectively. Cysteine and glutathione contents of the transformants were 3.4, 1.75 and 2.4, 2.89 times higher than those of wild type, respectively. Sulfate uptake efficiency of the transformants, grown in the presence of 1,000 mg/l sulfate was 2.8 and 2.0 times higher than those of wild type, respectively. Transformants and wild type absorbed sulfate at 10.6, 7.6 and 3.8 mg/g fresh weight, respectively, in tested condition. There was no difference of phenotype and growth rate observed between transformants and wild type when grown in either normal condition or in the presence of 1,000 mg/l sulfate. The transformants appeared to be more resistant to sodium sulfide than wild type.

Key words: Phytoremediation, sulfate, *Ipomoea aquatica*, cysteine synthase and *Oryza sativa*

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การกำจัดซัลเฟตด้วยผักบุ้งจีน *Ipomoea aquatica* Forsk. ดัดแปลงพันธุ์ที่มียีนซีสเตอีนซินเตสจากข้าวจ้าว *Oryza sativa* L.

อังคณา โพธิ์ไกร อัญชริดา อัครจรัลญา สุพัฒน์ เจริญพรวัฒนา ณัฐชนัญ ลีพิพัฒน์ไพบูลย์ ทัสซึโอะ นาคามูระ ยูเบะ ยามากูชิ อะซึฮิโกะ ชินมิโอะ และ ฮิโรชิ ซาโอะ (2549)
วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **31(1)**

ถ่ายโอนยีนระบุรหัสซีสเตอีนซินเตสของข้าวจ้าว *(Oryza sativa* cv. Nipponbare) ไอโซฟอร์มที่พบในโซโตพลาสซึม (ยีน *rcs*1) เข้าสู่ผักบุ้งจีนโดยวิธีการใช้ *Agrobacterium tumefaciens* EHA 101 ที่มีพลาสมิด pBIH1-IG(SX)-RCS1 พบว่าชิ้นส่วนใบเลี้ยง (cotyledon explant) จำนวน 908 ชิ้น ได้ต้นอ่อนที่งอกขึ้นมา 240 ต้น มีเพียง 2 ต้นที่สามารถต้านต่อสารปฏิชีวนะไฮโกรมัยซิน 25 ไมโครกรัมต่อมิลลิลิตร และให้ผลิตภัณฑ์จากกระบวนการ PCR ที่มีขนาดเท่ากับยีน *rcs*1 เมื่อนำดีเอ็นเอมาตรวจหายีน *rcs*1 โดยวิธี PCR เรียกผักบุ้ง 2 ต้นนี้ว่า ผักบุ้งทรานสฟอร์แมนท์พันธุ์หมายเลข 2 และ 4 กิจกรรมของซีสเตอีนซินเตสของผักบุ้งทรานสฟอร์แมนท์สูงกว่าผักบุ้งพันธุ์เดิม 3.4 และ 3.0 เท่า ปริมาณกรดอะมิโนซีสเตอีน และปริมาณกลูตาไธโอนของผักบุ้งทรานสฟอร์แมนท์สูงกว่าผักบุ้งพันธุ์เดิม 3.4, 1.75 และ 2.4, 2.89 เท่าตามลำดับ เมื่อปลูกผักบุ้งในสภาวะที่มีซัลเฟต 1,000 มิลลิกรัมต่อลิตร ผักบุ้งทรานสฟอร์แมนท์มีประสิทธิภาพการดูดซับซัลเฟตสูงกว่าผักบุ้งพันธุ์เดิม 2.8 และ 2.0 เท่า ผักบุ้งทรานสฟอร์แมนท์พันธุ์หมายเลข 2, 4 และผักบุ้งพันธุ์เดิมดูดซับซัลเฟต 10.6, 7.6 และ 3.8 มิลลิกรัม/กรัมน้ำหนักสดตามลำดับ ลักษณะและอัตราการเจริญของผักบุ้งทรานสฟอร์แมนท์ไม่แตกต่างจากของผักบุ้งพันธุ์เดิมเมื่อเจริญในสภาวะปกติ และสภาวะที่มีซัลเฟตเข้มข้น 1,000 มิลลิกรัมต่อลิตร ผักบุ้งทรานสฟอร์แมนท์ทนต่อโซเดียมซัลไฟด์มากกว่าผักบุ้งพันธุ์เดิม

คำสำคัญ phytoremediation ซัลเฟต ผักบุ้ง ซีสเตอีนซินเตส ข้าว

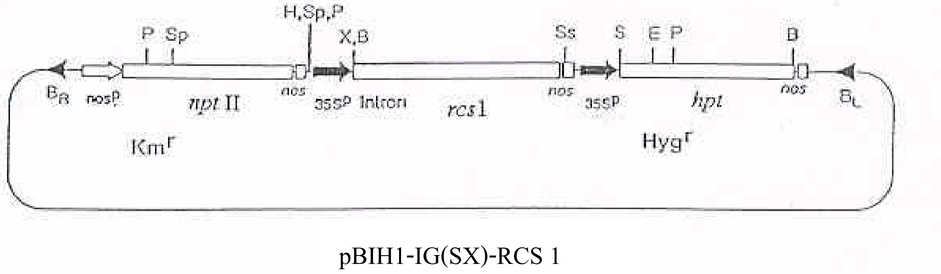
INTRODUCTION

Lignite coal is one of the main fuels used for generating electricity in Thailand. The water generated from the mining process at Mae Moh lignite mine has been found to contain a high concentration of sulfate ion (800-2,000 ppm). An attempt to remove sulfate ion was done using the wet land method. The water was siphoned through the anaerobic pond where sulfate ion was reduced to hydrogen sulfide by anaerobic bacteria, and the hydrogen sulfide was removed by ventilation. However, only 30% of the sulfate was removed by this method and the treated water still contained too high a concentration of sulfate to be released directly into the natural water resource. Hence, it was pooled in a big reservoir (biological pond) and its sulfate content was diluted by mixing with nearby natural water. Plants absorb sulfate from the environment and convert it into cysteine by the cysteine biosynthetic pathway.[1] Cysteine can serve as a substrate for all the molecules in plants containing – 2 valence sulfur. These include a wide range of compounds such as methionine, vitamins, coenzymes, secondary metabolites and proteins. It has been reported that cysteine biosynthetic pathway involves several enzymatic reactions.[2,3] The crucial step is the reduction of sulfate to sulfite and then to sulfide through the sulfate reduction pathway. The final step of cysteine biosynthesis is the incorporation of sulfide into cysteine. The reaction is catalyzed by cysteine synthase (O-acetyl-L-serinethiol-lyase, EC 4.2.99.8), which uses sulfide and O-acetyl-L-serine as the substrate. *Ipomoea aquatica* Forsk., a fast growing aquatic plant, grows well in the biological pond. Thus, the *Ipomoea aquatica* Forsk. which is able to overexpress an enzyme in the cysteine biosynthetic pathway may be promising as a sulfate absorber and converter in the biological pond. In this present study, transgenic *Ipomoea aquatica* Forsk. overexpressing cysteine synthase gene was constructed and its sulfate absorption efficiency was studied.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Plasmid pBIH1-IG containing β-glucuronidase (*gus*), hygromycin phosphotransferase *(htp*) and neomycin phosphotransferase II (*nptII*) genes in the T-DNA region[4] which *Xho*I site was modified to *Sac*I site by Nakamura *et al.*[5] was used as a vector. Double-digestion of the modified plasmid pBIH1-IG or plasmid pBIH1-IG (SX) by *Xba*I and *Sac*I resulted in *gus* deletion. The *rcs*1, cytosolic cysteine synthase gene of rice (*Oryza sativa* cv. Nipponbare),[5] was ligated to pBIH1-IG (SX) previously digested by *Xba*I and *Sac*I resulting in pBIH1-IG (SX)-RCS1 (Figure 1). A*grobacterium tumefaciens* EHA 101[6] harbouring pBIH1-IG (SX)-RCS1 was used throughout the experiments. A single colony of *A. tumefaciens* EHA101 harboring pBIH1-IG (SX)-RCS1 was inoculated into 10 ml YEP medium (10 g/l Bactopeptone, 10 g/l yeast extract, 5 g/l sodium chloride) supplemented with 50 mg/l kanamycin and 50 mg/l hygromycin in 125 ml flask and incubated at 28^{o}C with aeration by shaking at 150 rpm for 24 hrs. Then 0.5 ml was transferred to 50 ml of the same medium in a 250 ml flask and incubated at the same condition for 24 hrs (late log phase) and used for transformation.



pBIH1-IG(SX)-RCS 1

Figure 1. Plasmid pBIHI-IG(SX)-RCS1
Restriction enzyme abbreviation : P (*Pst*I), Sp (*Spn*I),
H (*Hind*III), B (*Bam*HI), S (*Sal*I), Sn (*Sna*BI), X (*Xba*I), Ss (*Sac*I), E (*Eco*RI).

Plant transformation

Seeds of *Ipomoea aquatica* Forsk. purchased from Chia Tai Co. Ltd., Thailand were surface sterilized with 70% ethanol for 2 min, 1% NaOC1 for 30 min and then rinsed five times with sterile distilled water. After final immersion of the seeds in sterile distilled water for 30 min, they were transferred to MS solid medium[7] supplemented with 30 g/l sucrose and 2.5 g/l gellan gum, pH 5.8 for germination. Modified MS medium consisting of half-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, pH 5.8[8] was used as the basal medium for plant tissue culture. The incubation conditions were 25°C under 16-hr photo period at light intensity of 24 μmol $m^{-2}s^{-1}$ (white fluorescent tube). Cotyledon segments including petiole-like cotyledon base[9] excised from one week-old seedlings were used for plant gene transformation. Two hundred cotyledon segments were suspended in 20 ml of MS medium containing $9.0x10^6$ cells/ml of late log phase *Agrobacterium tumefaciens* and 50 μM acetosyringone (3', 5'-Dimethoxy-4'-hydroxy-acetophenone; Aldrich Chem Co. Ltd., Germany) in 50 ml plastic tube for 1 hr with shaking at 80 rpm. The cotyledon segments, filtered through sterile stainless mesh and blotted dry on sterile filter paper, were cultured on MS solid medium containing 50 μM acetosyringone and incubated at 25°C in the dark for 3 days. The infected cotyledon segments washed with sterile distilled water containing 300 mg/l cefotaxime (Wako Co. Ltd., Japan) twice and then blotted dry on sterile filter paper, were cultured on modified MS solid medium containing 10 μM TDZ (thidiazuron) (Wako Co. Ltd., Japan) and 300 mg/l cefotaxime and incubated at the same above condition for one month with transferring to new medium at two week intervals. Two cm-height regenerated shoots were transferred to modified MS solid medium containing 10 μM TDZ, 25 mg/l hygromycin and 300 mg/l cefotaxime and incubated at the same above condition for one month.[10]

Plantlet transfer

The regenerated shoots surviving after 1 month on modified MS solid medium containing 10 μM TDZ, 25 mg/l hygromycin and 300 mg/l cefotaxime were transferred to hormone-free MS solid medium containing 300 mg/l cefotaxime for root generation. Plantlets with well developed shoot and root were transferred to half-strength MS inorganic salts without supplementation with any organic compound and incubated at the same above condition to acclimate for one week. Then, plantlets were transferred to glasshouse condition.

PCR analysis of *rcs*1

Genomic DNA extracted from leaves of putative transformed plantlets by the modified method described by Edwards *et al.*[11] was used as a template for *rcs*1 gene amplification. The sequence of oligonucleotide primers were 5'TGTCAGATCGATTCCTGACG-3' (*rcs*1-1) and 5'-TGATGGACTGGAAGAGCACC-3' (*rcs*1-2) located at the base-position 49-68 and 988-1007 of *rcs*1 sequence, respectively. The PCR was performed with a DNA thermal cycler (GeneAmp 2400, Perkin Elmer, USA) under the following condition: 94°C for 1 min, followed by 30 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 1.30 min with a final 5 min period at 72°C.

Cysteine synthase activity assay

Ten milligrams fresh weight of leaves were ground in a liquid nitrogen cooled 1.5 ml Microfuge tube with 600 μl of extraction buffer (50 mM Tris-Cl buffer pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT and 0.1% (w/v) Triton X-100) and centrifuged at 10,000 *g*, at 4°C for 30 min. The supernatant was kept at 0°C and used as crude enzyme solution. Cysteine synthase activity was determined from the amount of cysteine synthesized by using sodium sulfide and O-acetyl L-serine as substrate, and 5' pyridoxal as co-factor. The amount of cysteine synthesized was measured by the method described by Gaitonde.[12] Crude enzyme solution containing 1 microgram protein was added to 100 μl (final volume) of reaction mixture composed of 50 mM Tris-HCl buffer pH 8.0, 5 mM DTT, 5 μM pyridoxal, 10 mM O-acetyl L-serine, 2 mM sodium sulfide in a 1.5 ml Microfuge tube, mixed, and kept at room temperature for 15 min. The reaction was stopped by addition of 20 μl of 20% trichloroacetic acid. After sequential addition of 100 μl of concentrated acetic acid and 200 μl of ninhydrin solution (250 mg ninhydrin in 6 ml of concentrated acetic acid and 4 ml of concentrated HCl), the reaction mixture was heated in boiling water for 10 min then immediately cooled in iced water. Five hundred microliters of absolute ethanol was added and then the absorbance at 560 nm was measured. The reaction mixture stopped at the zero time by addition of 20% trichloroacetic acid was used as control. Protein concentration of crude enzyme was quantified by the method described by Bradford.(13) Three independent plants of each line were analyzed and triplicate measurement was done for each sample.

Cysteine and glutathione content assay

Cysteine and glutathione were quantified by the method described by Noctor and Foyer.(14) Leaf samples (100 mg) were frozen in liquid nitrogen and ground in 600 µl of solution containing 0.1 N HCl and 1 mM EDTA. After centrifugation at 10,000 *g*, 4°C for 30 min, the supernatant was labeled with monobromobimane. Then 100 µl aliquot was mixed with 20 µl of 10 mM DTT, pH was adjusted to 8.0 and immediately mixed with 20 µl of 1 mM monobromobimane in acetonitrile. The mixture was kept in the dark at room temperature for 15 min and mixed with 0.6 ml of 10% acetic acid and kept on ice for 5 min. Supernatant obtained from centrifugation at 5,000 *g*, 4°C for 2 min was analyzed by HPLC (HP 1100, Hewlett Packard, USA) equipped with Hypersil ODS 5 µm column (4 mm x 125 mm) (Lichrospher-100, Hewlett Packard, USA) and fluorometer fitted with 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 time of cysteine and glutathione was 4.0 and 5.0 min, respectively. Three independent plants of each line were analyzed and triplicate measurement was done for each sample.

Sulfate uptake assay

Ipomoea aquatica Forsk. was cultivated in MS medium containing 1,000 mg/l sulfate and 300 mg/l cefotaxime for 2 weeks. After 2 weeks, *Ipomoea aquatica* Forsk. was washed with distilled water, blotted dry and weighed. The amount of sulfate residue in MS medium was analyzed by HPLC (ED50 Electrochemical detector, Dionex Co. Ltd., USA) equipped with IonPac AS11 column (4 x 250 mm) and conductivity detector. The sample was eluted at 1 ml/min flow rate by 12 mM potassium hydroxide. Retention time of sulfate was 4.23 min. Three independent plants of each line were tested and triplicate measurement was done for each sample.

Sodium sulfide tolerance

Transformants, IRCS2 and IRCS4, and wild type were treated with sodium sulfide by culturing in MS medium containing 0.25 mM sodium sulfide. After 1 month, the percentage of fresh weight increase of the transformants and wild type were compared. Three independent plants of each line were tested and triplicate measurement was done for each sample.

RESULTS AND DISCUSSIONS

Transformation of *Ipomoea aquatica* Forsk.

From 908 cotyledon segments infected with *Agrobacterium tumefaciens* harbouring pBIH1-IG(SX)-RCS1, 240 regenerated shoots were obtained. Among these regenerated shoots, 2 shoots designated as IRCS2 and IRCS4 were tolerant to 25 µg/ml hygromycin. PCR analysis of genomic DNA extracted from leaves of these 2 putative transformants revealed an amplified fragment (958 bp) coincided with the DNA fragment obtained when using *rcs*1 fragment as a template and using the specific oligonucleotide primers, *rcs*1-1 and *rcs*1-2 (Figure 2). No PCR product was detected when genomic DNA of wild type was used as a template. This indicated low homology between *Oryza sativa* L. and *Ipomoea aquatica* Forsk. cysteine synthase gene.

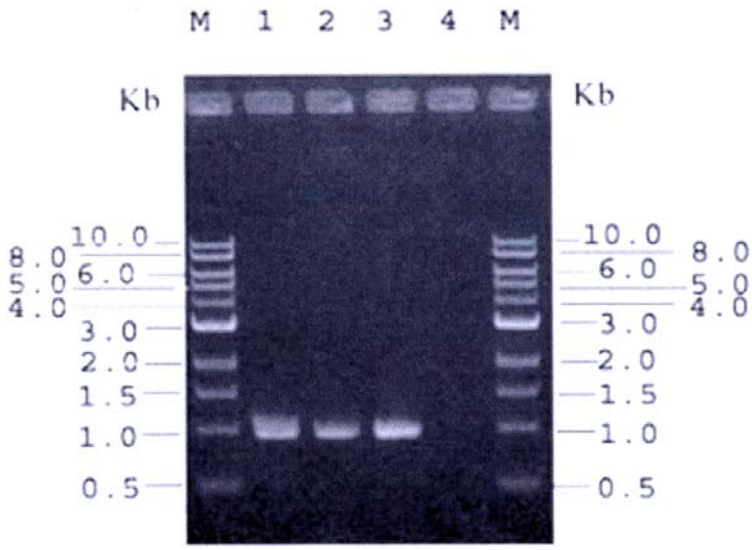


Figure 2. PCR analysis of genomic DNA extracted from leaves of *Ipomoea aquatica* Forsk. DNA ladder, 0.5 to 10 kb (M); plasmid pBIH1-IG(SX)-RCS1, positive control (1); DNA extracted from transformants, IRCS2, IRCS4 and wild type, respectively (2, 3, 4).

Cysteine synthase activity, cysteine and glutathione content

Cysteine synthase activity of the transformants, IRCS2 and IRCS4, was 0.45 and 0.4 units/mg protein or 3.4 and 3.0 times higher than those of wild type, respectively (Figure 3). Cysteine and glutathione contents of the transformants and wild type were compared. Cysteine content of the transformants was 27.8 and 14.5 pmole/mg leaf fresh weight or 3.4 and 1.75 times higher than wild type, respectively. Glutathione content of the transformants was 39.8 and 48.1 pmole/mg leaf fresh weight or 2.4 and 2.89 times higher than wild type, respectively (Figure 4).

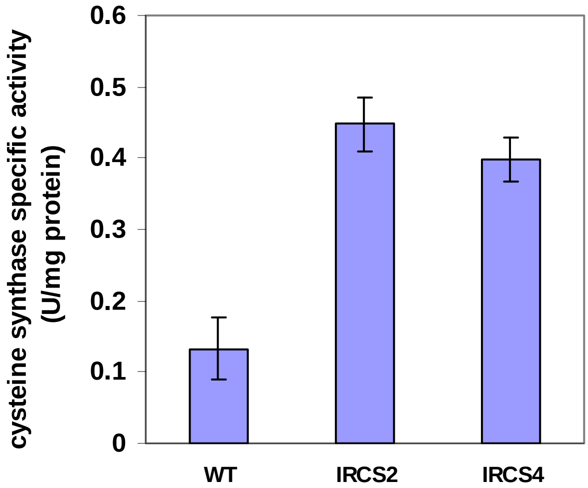


Figure 3. Comparison of cysteine synthase activity of wild type (WT) and the transformants, IRCS2 and IRCS4.

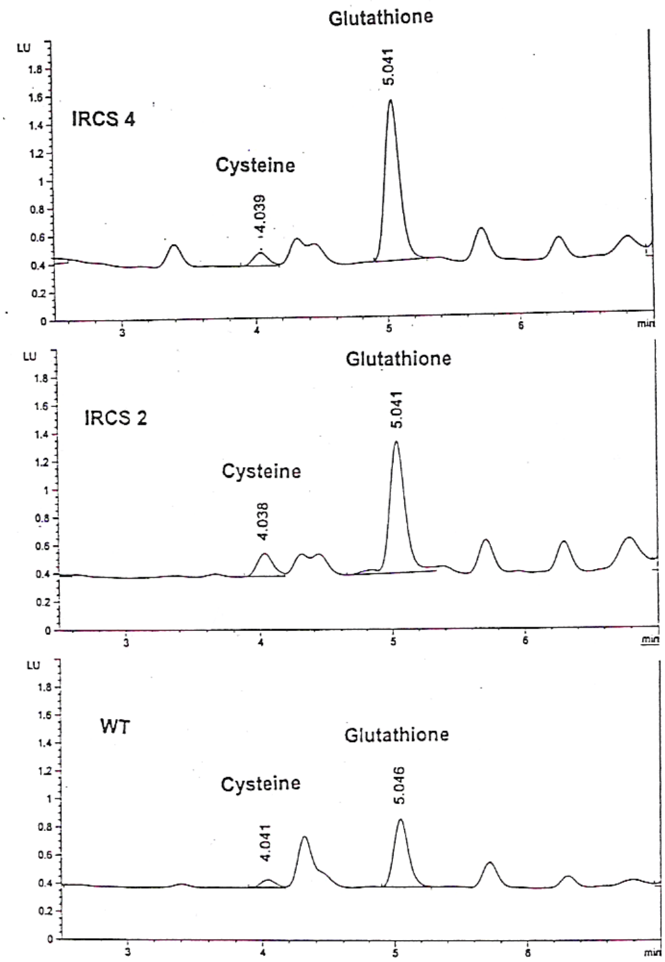


Figure 4. Comparison of cysteine and glutathione content of the transformants, IRCS2 and IRCS4, and wild type (WT).

Sulfate uptake

Ipomoea aquatica Forsk. was cultivated in MS medium containing 1,000 mg/l sulfate and 300 mg/l cefotaxime for 2 weeks, then the amount of sulfate uptake was analyzed. Sulfate uptake efficiency of the transformants was 2.8 and 2.0 times higher than those of wild type (Figure 5). IRCS2 and IRCS4, and wild type absorbed 10.6, 7.6 and 3.8 mg sulfate/g fresh weight, respectively in tested condtion. Phenotype and growth rate of the transformants and wild type grown in the presence of 1,000 mg/l sulfate or normal condition were not different. Overexpression of *Arabidopsis thaliana* ATP sulfurylase in tobacco (*Nicotiana tobaccum*) had no effect on sulfate uptake.[15] However, overexpression of rice cysteine synthase gene in the transformants resulted in the increase of sulfate uptake. The results suggested that cysteine synthase is a key step enzyme of the sulfate assimilation pathway in plants.

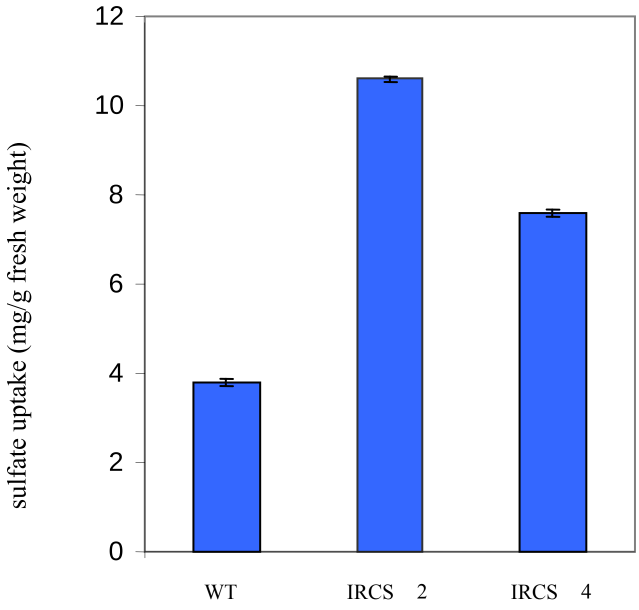


Figure 5. Comparison of sulfate uptake of wild type (WT) and the transformants, IRCS2 and IRCS4.

Sodium sulfide tolerance

After culturing for 1 month in the presence of 0.25 mM sodium sulfide, fresh weight increase of the transformants and wild type plants were compared. Percentage of fresh weight increase of the transformants in the presence of 0.25 mM sodium sulfide was 3.7 and 5.6 times higher than wild type (Figure 6). The results showed that the transformants were more resistant to sodium sulfide than wild type. Glutathione is a tripeptide molecule composed of glutamate, cysteine and glycine. Glutathione biosynthesis is strongly regulated by the cysteine availability.(16) Noij *et al.*(17) reported that overexpression of cysteine synthase caused the increase of glutathione and cysteine contents in tobacco (*Nicotiana tabacum*). Phytochelatins, of the basic structure $(\gamma\text{-Glu-Cys})_n$-Gly (n = 2-11), synthesized from glutathione when plants are exposed to heavy metals, play an important role in detoxification of toxic metals such as cadmium and arsenic.(18) Thus, the transgenic plants overproduce cysteine and glutathione and have a potential to be heavy metal tolerant plants.

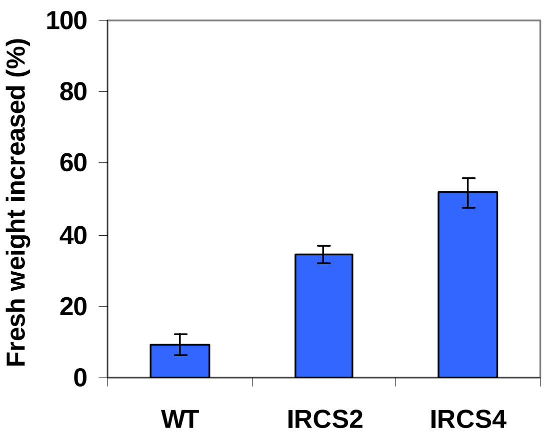


Figure 6. Effect of 0.25 mM sodium sulfide on percentage of fresh weight increase of *Ipomoea aquatica* Forsk., wild type (WT); transformants, IRCS2 and IRCS4.

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