

Expression of a Soybean (*Glycine max* [L.] Merr.) Seed Storage Protein Gene in Transgenic *Arabidopsis thaliana* and Its Response to Nutritional Stress and to Abscisic Acid Mutations¹

Satoshi Naito*, Masami Yokota Hirai, Mitsuo Chino, and Yoshibumi Komeda

Molecular Genetics Research Laboratory, University of Tokyo, Hongo Tokyo 113, Japan (S.N., Y.K.); Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Yayoi, Tokyo 113, Japan (M.Y.H., M.C.); and Department of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan (S.N.)

Among the three subunits of β -conglycinin, the 7S seed storage protein of soybean (*Glycine max* [L.] Merr.), expression of the β subunit gene is unique. Accumulation of the β subunit is enhanced in sulfate-deficient soybean plants, and its mRNA levels increase when abscisic acid (ABA) is added to the in vitro cotyledon culture medium. Transgenic *Arabidopsis thaliana* lines carrying a gene encoding the β subunit was constructed and grown under sulfate deficiency. Accumulation of both β subunit mRNA and protein were enhanced in developing *A. thaliana* seeds. Accumulation of one of the *A. thaliana* seed storage protein mRNAs was also enhanced by sulfate deficiency, although the response was weaker than that observed for the soybean β subunit mRNA. When the *aba1-1* or *abi3-1* mutations were crossed into the transgenic *A. thaliana* line, accumulation of the β subunit was significantly reduced, whereas accumulation of the *A. thaliana* seed storage protein was not greatly affected. These results indicate that soybean and *A. thaliana* share a common mechanism for response to sulfate deficiency and to ABA, although the sensitivity is different between the species. The transgenic *A. thaliana* carrying the β subunit gene of β -conglycinin will be a good system to analyze these responses.

The 7S ssp of soybean (*Glycine max* [L.] Merr.), β -conglycinin, comprises primarily three subunits, designated α' (76 kD), α (72 kD), and β (53 kD) (Derbyshire et al., 1976). The genes encoding these three subunits are all expressed during the middle and latter stages of seed development. However, detailed analyses of subunit gene expression revealed variation in temporal (Gayler and Sykes, 1981; Meinke et al., 1981; Naito et al., 1988) and spatial (Ladin et al., 1987) patterns of expression during soybean seed maturation. Nutritional stresses of whole plants such as sulfate deficiency or potassium deficiency change the accumulation of the subunit proteins (Gayler and Sykes, 1985). The addition of Met (Holowach et al., 1984, 1986) as well as ABA (Bray and

Beachy, 1985) has also been found to alter the subunit gene expression in soybean cotyledon cultures.

Gayler and Sykes (1985) reported increased accumulation of β -conglycinin in sulfate-deficient soybean plants and diminished quantities of the 11S protein (glycinin), another major ssp. Among the subunits of β -conglycinin, the β subunit responds to sulfate deficiency most dramatically and accumulates to levels 3-fold higher than when grown under normal conditions. Glycinin is rather rich in sulfur amino acids, whereas β -conglycinin is poor in sulfur amino acids. Among the subunits of β -conglycinin, the β subunit contains the least sulfur amino acids and bears only one Cys and no Met residue in its mature form (Coates et al., 1985). Thus, as a consequence of above-mentioned response versus sulfate deficiency, total protein content of soybean seed is kept fairly constant at the expense of sulfur-rich glycinin (Gayler and Sykes, 1985). However, the mechanism by which β subunit gene expression increases is not understood.

Genomic clones carrying the α' or β subunit gene of β -conglycinin have been isolated and were used to construct transgenic tobacco and petunia plants (Chen et al., 1989). Temporal regulation of these genes in transgenic petunia is similar to that observed in soybean (Naito et al., 1988). We recently found that β subunit mRNA accumulation is diminished by the addition of Met to in vitro cultures of transgenic petunia capsules. Furthermore, as in soybean, β subunit accumulation increased when grown under sulfate deficiency in transgenic petunia (Fujiwara et al., 1992). The β subunit gene has also been shown to be positively regulated by ABA in an in vitro soybean cotyledon culture system. Bray and Beachy (1985) reported that addition of ABA to in vitro cotyledon culture medium enhances β subunit gene expression, whereas α' and α gene expression are not affected. Similar experiments in *Brassica napus* have shown ssp mRNA accumulation to be enhanced by exogenously supplied ABA in cultured embryos (Finkelstein et al., 1985; DeLisle and Crouch, 1989).

In this report, we describe the construction of transgenic *A. thaliana* carrying the β subunit gene of β -conglycinin. We

¹ This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan to Y.K., M.C., and S.N. and by a grant from The Asahi Glass Foundation to S.N.

* Corresponding author; fax 81-11-746-0208.

Abbreviation: ssp, seed storage protein(s).

found expression of this gene very responsive to sulfate deficiency in the transgenic lines, whereas the endogenous *A. thaliana* ssp mRNA accumulation was less variable in response to sulfur nutrition. Finally, we constructed transgenic *A. thaliana* lines that carry the β subunit gene and are also homozygous for one of several mutations that affect either ABA biosynthesis (*aba1*; Koornneef et al., 1982) or physiological responses to ABA (*abi1*, *abi2*, and *abi3*; Koornneef et al., 1984). In this report, we describe the effects of these mutations on β subunit expression.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (L.) Heynh. wild type, Col-0 (Columbia ecotype), was obtained from Dr. G.P. Rédei and was used to construct transgenic plants. *A. thaliana* lines carrying the *abi1-1* (isolation number A II), *abi2-1* (E II), *abi3-1* (C IV) alleles (Koornneef et al., 1984), the *aba1-1* (A26) allele (Koornneef et al., 1982), and the parental line, *La-er*, the Landsberg ecotype carrying the *erecta* mutation, were obtained from Dr. M. Koornneef.

All plants except those used in sulfate-deficiency experiments were sown in rockwool bricks (3 × 3 × 4 cm; Nittobo Co., Tokyo, Japan) and transferred to pots containing vermiculite when the seedlings were at the four-leaf stage. The plants were grown under continuous white fluorescent light at about 400 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 22°C and were irrigated twice a week with MGRL medium (Fujiwara et al., 1992) containing 1.75 mM sodium phosphate (pH 5.8), 1.5 mM MgSO_4 , 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 3.0 mM KNO_3 , 67 μM Na_2EDTA , 30 μM H_3BO_3 , 10.3 μM MnSO_4 , 8.6 μM FeSO_4 , 1.0 μM ZnSO_4 , 1.0 μM CuSO_4 , 130 nM CoCl_2 , and 24 nM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. Under these conditions, *A. thaliana* seeds were fully mature at about 16 DAF.

The plants used in sulfate-deficiency experiments were grown in rockwool bricks (10 × 10 × 12 cm) under continuous white fluorescent light at about 400 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 22°C. They were irrigated twice a week with either MGRL medium (control) or a sulfate-deficient medium, which had the same composition as MGRL medium except that MgSO_4 was omitted and 1.5 mM MgCl_2 was included.

Construction of Transgenic *A. thaliana* Lines Carrying the β Subunit Gene

A 4.2-kb DNA fragment encoding the β subunit of β -conglycinin was excised from pGmg91 (Tierney et al., 1987) by digestion with *Hind*III and was recloned into pMON410 (Rogers et al., 1987; obtained from Monsanto Co., St. Louis, MO) at the single *Stu*I site to obtain pSN211. pMON410 and pSN211 were introduced into an *Agrobacterium tumefaciens* strain by triparental mating.

Transformation of *A. thaliana* Col-0 was carried out by the leaf disc method of Lloyd et al. (1986) using hygromycin resistance as a selection marker. Two independently transformed lines, SNT8 and SNT16, were recovered after transformation with pSN211. A transformed control line, SNT11, was also recovered after transformation of Col-0 with pMON410. The transformation vector, pMON410, carries genes that confer both kanamycin and hygromycin resistance

to stable transformants. Those lines that are homozygous for kanamycin and hygromycin resistance were established in the subsequent generations.

Genetic Construction of Mutant Lines Expressing the β Subunit

SNT8 (male parent) was crossed to *La-er* lines (female parent) carrying one of the following mutations: *abi1-1*, *abi2-1*, *abi3-1*, and *aba1-1*. The F_1 progenies were crossed to the same female parent twice more. Lines that were homozygous for kanamycin resistance, *erecta*, and the *aba1-1*, *abi1-1*, or *abi2-1* allele were established in the F_3 generation. The SNT8 insertion appeared to be linked to the *ABI3* locus; therefore, the lines homozygous for kanamycin resistance, *erecta*, and *abi3-1* were established in the F_4 generation. For each of the *aba* and *abi* alleles, two lines were established from independent parental crosses. A control line carrying the kanamycin resistance marker and the *erecta* allele was constructed by crossing SNT8 to *La-er*.

DNA Extraction and Southern Hybridization

General DNA manipulations were conducted according to the method of Sambrook et al. (1989). DNA was extracted as described by Leutwiler et al. (1984) from 1 g of rosette leaves that were harvested from 3-week-old plants grown in rockwool bricks. The use of rockwool alleviates the contamination of soil material. DNA (1 μg) was digested with *Hind*III, size fractionated by agarose gel electrophoresis (0.7%), and transferred to a nylon membrane (GeneScreen Plus; DuPont, Wilmington, DE). Southern hybridization was carried out as recommended by the manufacturer. A 4.2-kb *Hind*III fragment carrying the gene encoding the β subunit of β -conglycinin was used as a probe. ^{32}P -labeled probes were prepared by random primer labeling.

Extraction of Seed Protein and Immunoblot Analysis

Protein was extracted from mature seeds as described by Naito et al. (1988), except that 20 μL of extraction buffer was used per mg of seed. SDS-PAGE (15%) and immunoblot analysis with a rabbit anti- β -conglycinin antibody were carried out as previously described (Naito et al., 1988). Defatted soybean (cv Toyosuzu) seed protein extract was used as a reference sample. Protein mol wt markers were purchased from Bio-Rad, and the Bio-Rad protein assay was used to determine protein concentration. Proteins were stained with Coomassie brilliant blue R-250 (Sigma).

Total RNA Extraction and Northern Hybridization

Developing fruits were staged by marking flowers with colored threads on the day of flower opening. Immature fruits were harvested, frozen in liquid nitrogen, and stored at -80°C until used. Total RNA was prepared by grinding 12 fruits in an ice-cold mortar and pestle with 300 μL each of extraction buffer (Naito et al., 1988) and phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The slurry was transferred to microfuge tubes and homogenized thoroughly with a Teflon pestle. This step gives a better yield of total

RNA, and we observed less variation in the yield of total RNA among experiments, although longer RNA species are sheared to some extent. The RNA was purified as described by Naito et al. (1988), size fractionated on 1.2% agarose gels in the presence of formaldehyde, and transferred to nylon membranes (GeneScreen Plus). Northern hybridization was carried out as recommended by the manufacturer. Accumulation of *A. thaliana* ssp mRNA was analyzed with genes encoding the 12S ssp, CRA, cloned in sAT2015 (Pang et al., 1988), and the 2S ssp, AT2S1, cloned in pEK1 (Krebbers et al., 1988). A *Xho*I digest of sAT2015 and a 0.7-kb *Hind*III-*Eco*RI fragment of pEK1 were used as probes. 32 P-labeled DNA probes were prepared by random primer labeling. Before the nylon membrane was reused, the membrane was regenerated as recommended by the manufacturer. Total RNA prepared from soybean (cv Provar) seeds harvested 35 DAF was used as a reference sample. Accumulation of β subunit mRNA reaches the maximum level 35 DAF in soybean (Naito et al., 1988).

Comparison of Expression Level

Experiments were done at least in duplicate, and autoradiograms and SDS-polyacrylamide gels stained with Coomassie brilliant blue R-250 were quantified using a CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan). In northern analysis, smears were also included. Autoradiograms used for quantification were exposed several times and analyzed separately.

RESULTS

Characterization of Transgenic *A. thaliana* Carrying the Gene Encoding the β Subunit of β -Conglycinin

Two transformed lines, SNT8 and SNT16, were obtained by transformation of Col-0 strains with pSN211, which carries the β subunit gene of β -conglycinin. The β subunit gene includes 1.1 and 1.2 kb of 5' and 3' flanking sequences, respectively. A transformed control line, SNT11, was obtained by transformation with the vector, pMON410. The results presented in this report were obtained after establishing lines that are homozygous for the introduced gene.

Copy numbers of the introduced gene were analyzed by quantitative Southern blot (Fig. 1a). The estimated copy numbers of the β subunit gene carried by SNT8 and SNT16 were 1 and 3, respectively. We also analyzed the segregation of the kanamycin resistance marker in SNT8 and SNT16 after each line was crossed to the untransformed line, Col-0. The results indicated that kanamycin resistance in each line segregated as a single locus that segregates 3:1 in the F_2 generation (61:18, $\chi^2 = 0.21$ for SNT8; 77:24, $\chi^2 = 0.083$ for SNT16).

Mature seeds were collected from Col-0, SNT8, SNT16, and SNT11 and analyzed by immunoblot for accumulation of the β subunit. As shown in Figure 1b, both SNT8 and SNT16 accumulated a protein of the same mol wt as that of the β subunit of β -conglycinin, which was not present in seeds of Col-0 or SNT11. SNT16, which is estimated to carry three copies of the β subunit gene, accumulated 2- to 3-fold more β subunit than did the single-copy transformant, SNT8.

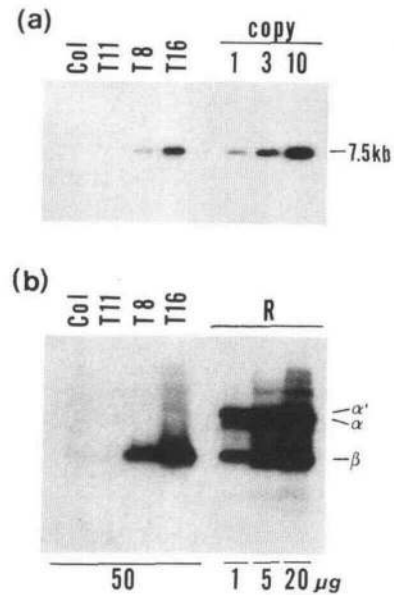


Figure 1. Characterization of transgenic plants. a, Determination of transgene copy number. Total DNA (1 μ g) was digested with *Hind*III and analyzed by Southern hybridization using the β subunit gene labeled with 32 P as a probe. Copy number references were reconstituted by mixing appropriate amounts of *Hind*III-digested pSN211 DNA with 1 μ g of *Hind*III-digested untransformed Col-0 DNA. The genome size of *A. thaliana* was taken as 70,000 kb per haploid genome (Meyerowitz and Pruitt, 1985). By *Hind*III digestion pSN211 gives a 7.5-kb DNA fragment carrying the β subunit gene. b, Detection of the β subunit protein in seeds. *A. thaliana* seed protein (50 μ g) was subjected to immunoblot analysis using an anti- β -conglycinin antibody, which reacts with all three subunits of β -conglycinin. Reference lanes (R) contain 1, 5, and 20 μ g of soybean seed protein. The migration positions of the α , α' , and β subunits are indicated. Col, Untransformed Col-0; T11, SNT11; T8, SNT8; T16, SNT16.

Expression of β Subunit Gene under Sulfate Deficiency

To determine whether the transgenic *A. thaliana* lines carrying the β subunit respond to sulfate deficiency, SNT8, SNT16, and Col-0 plants were irrigated with either control (1.5 mM sulfate) or sulfate-deficient (about 20 μ M sulfate) nutrient solution. The plants given 20 μ M sulfate showed no severe symptoms of sulfate deficiency, and there was no obvious difference in the time course of fruit maturation between plants grown under these two conditions. However, the rosette leaves of mature plants became yellow a few days earlier than those supplied with 1.5 mM sulfate.

As shown in Figure 2, mature seeds of both SNT8 and SNT16 grown on 20 μ M sulfate accumulated more of the β subunit than did the same lines supplied with 1.5 mM sulfate. When grown on 20 μ M sulfate, SNT8 and SNT16 accumulated approximately the same amounts of the β subunit. When grown on 1.5 mM sulfate, the single-copy line, SNT8, accumulated less of the β subunit than did the multicopy transformant, SNT16. Thus, approximately 3-fold higher levels of β subunit accumulation were observed in SNT8 when grown on 20 μ M sulfate, whereas the corresponding value for SNT16 was about a 1.5-fold increase.

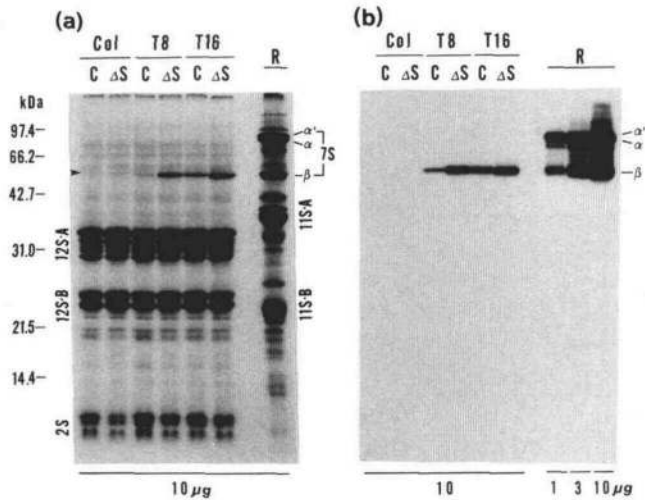


Figure 2. Accumulation of the β subunit protein in transgenic *A. thaliana* in response to sulfate deficiency. Protein extracts were prepared from mature seeds harvested from plants grown with either 1.5 mM (C) or 20 μ M (Δ S) sulfate. Seed protein extract (10 μ g) was subjected to 15% SDS-PAGE and either stained with Coomassie brilliant blue R-250 (a) or visualized by immunoblot analysis (b). Seed proteins from two transgenic lines, SNT8 (T8) and SNT16 (T16), as well as an untransformed control line, Col-0 (Col), were analyzed. Reference lanes (R) contain 10 μ g (a) and 1, 3, and 10 μ g (b) of soybean seed protein. The following ssp as well as size markers are all indicated: α , α' , and β subunits of β -conglycinin (7S), the acidic (11S-A), and basic (11S-B) subunits of soybean glycinin, the acidic (12S-A) and basic (12S-B) subunits of 12S ssp of *A. thaliana*, and the 2S ssp (2S) of *A. thaliana*. The arrowhead in a indicates a minor protein in the untransformed wild type (Col), which is enhanced under sulfate deficiency.

To determine whether or not the increased accumulation of the β subunit in transgenic *A. thaliana* under sulfate deficiency is regulated at the level of mRNA accumulation, we analyzed mRNA accumulation in developing fruits of SNT8 and SNT16. Figure 3 shows accumulation of the β subunit mRNA in fruits of SNT8 and SNT16 harvested 12 DAF. The β subunit mRNA was undetectable in Col-0 fruits. Fruits of SNT16 grown in 1.5 mM sulfate accumulated about 3 times more β subunit mRNA than did SNT8 fruits. When grown in 20 μ M sulfate, SNT8 and SNT16 accumulated approximately the same amount of β subunit mRNA, which was about 700% and 200 to 300% of the mRNA levels observed in SNT8 and SNT16, respectively, grown in 1.5 mM sulfate. Thus, β subunit mRNA accumulation varies with the nutritional supply of sulfate.

Effects of Sulfate Deficiency on *A. thaliana* ssp

The major seed reserve proteins of *A. thaliana* are the 12S (related to cruciferin in *B. napus*) and 2S (related to napin in *B. napus*) proteins (Heath et al., 1986), which are encoded by small multigene families. Krebbers et al. (1988) have reported the isolation of four genes encoding the 2S ssp of *A. thaliana*. The coding regions of the genes, AT2S1 to AT2S4, are more than 80% homologous to one another, and all follow a similar

temporal pattern of mRNA accumulation in developing seeds (Guerche et al., 1990). Pang et al. (1988) reported three genes encoding the 12S ssp in the Columbia ecotype, CRA, CRB, and CRC, which are less closely related to one another than the genes encoding the 2S ssp.

We found no obvious difference in the accumulation of the 12S ssp of *A. thaliana* in either Col-0, SNT8, or SNT16 under the two growth conditions used. However, accumulation of the 2S ssp was reduced when grown on 20 μ M sulfate (Fig. 2a). We also observed increased accumulation of a minor protein in the extracts of untransformed Col-0 seeds produced by plants grown on 20 μ M sulfate (arrowhead in Fig. 2a).

Accumulation of major ssp mRNAs of *A. thaliana* was analyzed in fruits of SNT8 and Col-0 12 DAF. Figure 4 shows that there is no significant difference in the accumulation of the 2S ssp mRNA between the two conditions. However, there was a small increase in the accumulation of the 12S ssp mRNA under sulfate deficiency. Thus, although accumulation of *A. thaliana* ssp mRNA is not as responsive to sulfate deficiency as is β subunit mRNA, the level of at least one of the *A. thaliana* ssp mRNA does vary in response to sulfate deficiency.

Effects of *aba* and *abi* Mutations on β Subunit Expression

Transgenic *A. thaliana* lines homozygous for the β subunit as well as one of the *aba1-1*, *abi1-1*, *abi2-1*, or *abi3-1* mutations were constructed by genetic crosses, and the effect of these mutations on β subunit accumulation was analyzed. Comparison of transgene expression between independent transgenic lines is often complicated by variation in expression due to insertion of the transgene into different regions of the genome, or position effect. However, because the transgene in each of these four lines occupies the same

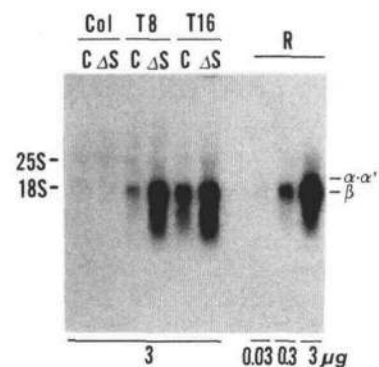


Figure 3. Accumulation of β subunit mRNA in response to sulfate deficiency. Untransformed Col-0 (Col), SNT8 (T8), and SNT16 (T16) were grown with either 1.5 mM (C) or 20 μ M (Δ S) sulfate, and total RNA was prepared from fruits harvested 12 DAF. Total RNA (3 μ g) was analyzed by northern hybridization using 32 P-labeled β subunit gene as a probe. Reference lanes (R) contain 0.03, 0.3, and 3 μ g of soybean seed RNA. The α , α' , and β subunit mRNA as well as the 25S and 18S rRNA are indicated. The β subunit gene has extensive homology to those encoding α and α' ; therefore, the β subunit gene probe cross-hybridizes with the α' and α subunit mRNA under standard hybridization conditions (Bray and Beachy, 1985).

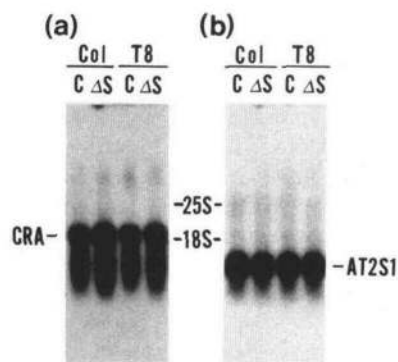


Figure 4. *A. thaliana* ssp mRNA accumulation in response to sulfate deficiency. Untransformed Col-0 (Col) and SNT8 (T8) were grown with either 1.5 mM (C) or 20 μ M (Δ S) sulfate, and total RNA was prepared from fruits harvested 12 DAF. Total RNA (1 μ g) was analyzed by northern hybridization using the gene encoding the *A. thaliana* 12S ssp labeled with 32 P as a probe (a). The membrane was rehybridized using the *A. thaliana* 2S ssp gene as a probe (b). The 12S ssp mRNA (CRA) and the 2S ssp mRNA (AT2S1) as well as the positions of 25S and 18S rRNA are indicated.

chromosomal position, position effect variation is not a concern when comparing gene expression between these lines.

As shown in Figure 5, introduction of the ABA-deficient mutation, *aba1-1*, reduced accumulation of the β subunit to about 30% of the wild-type level. The *aba1-1* mutant contains less than 5% of the wild-type level of ABA in seeds (Koornneef et al., 1982). This result indicates that the β subunit gene is responsive to ABA levels in vivo. Three classes of ABA-

insensitive mutations, *abi1* through *abi3*, have been isolated in *A. thaliana* (Koornneef et al., 1984). Introduction of *abi3-1* mutation reduced accumulation of the β subunit to about 7%, whereas the *abi1-1* and *abi2-1* mutations had little or no effect. It is also evident that there is a visible change in Coomassie brilliant blue R-250 staining pattern in the strains carrying the *abi3-1* mutation but not in the *abi1-1* or *abi2-1* mutation. Our results corroborate those of Finkelstein and Somerville (1990), who observed about 30% reduction in 12S protein accumulation in lines homozygous for the *abi3-1* mutation and no effect of the *abi1-1* or *abi2-1* mutations on ssp accumulation.

DISCUSSION

Gayler and Sykes (1985) reported a 3-fold increase in the accumulation of the β subunit of β -conglycinin in sulfate-deficient soybean plants. We found that β subunit protein as well as mRNA accumulation responds to sulfate deficiency in transgenic *A. thaliana* and that the magnitude of this response was no less than that observed in soybean plants. Furthermore, the increased accumulation of β subunit protein under sulfate deficiency paralleled that of the mRNA, suggesting that this response is regulated mainly at the level of mRNA accumulation. Analysis of β subunit mRNA accumulation in sulfate-deficient soybean plants has not been reported. We also analyzed expression of *A. thaliana* ssp genes in response to sulfate deficiency. Accumulation of 2S ssp mRNA did not change significantly, whereas 2S protein accumulation diminished under sulfate deficiency. This indicates that accumulation of 2S protein, which is rich in sulfur-containing amino acids (Guerche et al., 1990), is reg-

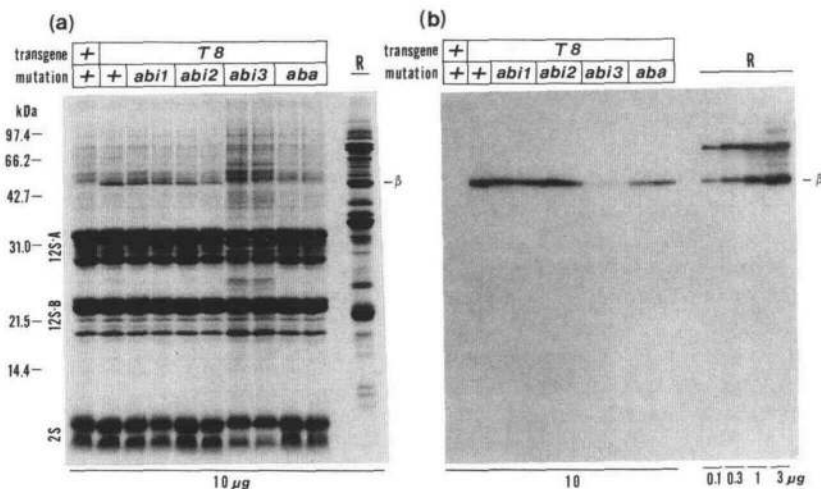


Figure 5. Accumulation of the β subunit protein in mutant lines of *A. thaliana*. *A. thaliana* lines that were homozygous for the SNT8 insertion (*T8*) and either *abi1-1* (*abi1*), *abi2-1* (*abi2*), *abi3-1* (*abi3*), or *aba1-1* (*aba*) mutation were grown with 1.5 mM sulfate, and protein extracts were prepared from mature seeds. Protein (10 μ g) was subjected to 15% SDS-PAGE and was stained with Coomassie brilliant blue R-250 (a) or subjected to immunoblot analysis (b). Reference lanes (R) contain 10 μ g (a) or 0.1, 0.3, and 3 μ g (b) of soybean seed protein. The migration positions of size markers and the β subunit (β) are indicated, as are the *A. thaliana* ssp: the 12S acidic (12S-A) and basic (12S-B) ssp and 2S ssp (2S). The genotype of the *A. thaliana* line being analyzed is indicated above the gel. The two lanes on the right of each panel contain extracts from untransformed Col-0 (+,+) and SNT8 crossed with *La-er* (*T8*,+). The remaining four genotypes in each panel are represented by extracts from two independently derived lines of the indicated genotype (*T8*, *abi1*; *T8*, *abi2*; *T8*, *abi3*; *T8*, *aba*).

ulated at a posttranscriptional step(s). Accumulation of 12S ssp mRNA, on the other hand, increased under sulfate deficiency.

The fact that both the soybean β subunit and *A. thaliana* 12S ssp mRNA accumulation increased under sulfate deficiency indicates that soybean and *A. thaliana* have a common mechanism for regulating gene expression in response to sulfate deficiency. We also observed an increase in the accumulation of a minor *A. thaliana* protein under sulfate deficiency (arrowhead in Fig. 2), providing further evidence for a common mechanism for response to sulfate deficiency. We previously reported that accumulation of the β subunit protein in transgenic petunia increases under sulfate deficiency (Fujiwara et al., 1992). Differential expression of ssp gene in response to sulfur availability has been reported not only in Leguminosae (Blagrove et al., 1976; Randall et al., 1979; Evans et al., 1985) but also in other dicotyledonous (Spencer et al., 1990) and in monocotyledonous plants (Shewry et al., 1983; Wrigley et al., 1984; Baudet et al., 1986). Taken together with the results presented here, these suggest that a wide range of plant species have similar mechanisms for responding to alterations in sulfur supply.

Bray and Beachy (1985) reported that addition of ABA to immature soybean cotyledon cultures enhanced the accumulation of β subunit mRNA without significantly affecting the accumulation of the α or α' subunit mRNAs. In *B. napus*, translation and accumulation of both cruciferin and napin mRNAs were shown to be enhanced by exogenously supplied ABA in in vitro embryo cultures (Finkelstein et al., 1985; DeLisle and Crouch, 1989). The results of these experiments have been difficult to interpret for two reasons. Excision and culture of embryos change the physiology of embryos regardless of whether or not ABA is supplied; thus, results obtained with such tissues may not faithfully reflect the in vivo system (Hughes and Galau, 1991). In addition to this, the physiological relevance of results obtained after exogenous application of a compound in tissues that are capable of producing normal levels of that compound is unclear. We tested the question of ABA effects on ssp synthesis in vivo by analyzing β subunit expression in transgenic *A. thaliana* lines deficient in ABA biosynthesis. The fact that accumulation of the β subunit was significantly reduced in *aba1-1* background indicates that the expression of β subunit gene is controlled by ABA in vivo.

The availability of *A. thaliana* mutants that do not respond to exogenous ABA (*abi1*, *abi2*, and *abi3* mutations) permitted us to analyze the effects of other mutations on β subunit gene expression. Nambara et al. (1992) found that a stringent allele of *abi3* causes dramatic reductions in 12S and 2S ssp mRNA and ssp accumulation. The mutant plants fail to complete seed development, remain green, and are desiccation intolerant. Koornneef et al. (1989) have shown that a line homozygous for *abi3-1*, a leaky allele of *abi3*, in an ABA-deficient background exhibits the same phenotypes as stringent alleles of *abi3*. However, the leaky allele of *abi3* alone has only minor effects on 12S ssp mRNA (Pang et al., 1988) and protein accumulation (Fig. 5a; Koornneef et al., 1984; Finkelstein and Somerville, 1990) and produces no gross developmental phenotypes. In our experiments, the same

leaky allele of *abi3* dramatically reduced accumulation of the β subunit in transgenic *A. thaliana*.

The *ABI3* gene has been cloned and sequenced and by homology appears to be a transcriptional activator (Giraudat et al., 1992), suggesting that *ABI3* regulates ssp gene expression via transcriptional activation. A possible explanation for the differential reduction between β subunit and *A. thaliana* ssp accumulation in the *abi3-1* background is that the promoter of the soybean gene may not function efficiently with *A. thaliana* transcription factors, so that a slight reduction in *ABI3* activity is sufficient to substantially reduce transcription of the heterologous gene but only slightly affecting transcription of the *A. thaliana* ssp genes. Another possibility is that the β subunit gene carries a *cis* element(s) that is more responsive to ABA than that carried by the *A. thaliana* genes.

In this report we presented evidence indicating that the gene encoding the β subunit of soybean β -conglycinin responds to sulfate deficiency, ABA level, and ABA sensitivity in transgenic *A. thaliana*. The advantageous nature of *A. thaliana* in genetic and molecular biological studies makes the transgenic *A. thaliana* harboring the β subunit gene an ideal system to further probe the plants' response to nutritional conditions and to ABA.

ACKNOWLEDGMENTS

We are grateful to Ms. Fumiko Nishigai for excellent technical assistance, to Ms. Kumi Fujiwara for general assistance, and to Dr. Toru Fujiwara, Ms. Kallie Keith, and Dr. Peter McCourt for valuable discussion and critical reading of the manuscript.

Received June 28, 1993; accepted October 20, 1993.

Copyright Clearance Center: 0032-0889/94/104/0497/07.

LITERATURE CITED

- Baudet J, Huet J-C, Jolivet E, Lesaint C, Mossé J, Pernellet J-C (1986) Changes in accumulation of seed nitrogen compounds in maize under conditions of sulphur deficiency. *Physiol Plant* **68**: 608-614
- Blagrove RJ, Gillespie JM, Randall PJ (1976) Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. *Aust J Plant Physiol* **3**: 173-184
- Bray EA, Beachy RN (1985) Regulation by ABA of β -conglycinin expression in cultured developing soybean cotyledons. *Plant Physiol* **79**: 746-750
- Chen Z-L, Naito S, Nakamura I, Beachy RN (1989) Regulated expression of genes encoding soybean β -conglycinins in transgenic plants. *Dev Genet* **10**: 112-122
- Coates JB, Medeiros JS, Thanh VH, Nielsen NC (1985) Characterization of the subunits of β -conglycinin. *Arch Biochem Biophys* **243**: 184-194
- DeLisle AJ, Crouch ML (1989) Seed storage protein transcription and mRNA levels in *Brassica napus* during development and in response to exogenous abscisic acid. *Plant Physiol* **91**: 617-623
- Derbyshire E, Wright DJ, Boulter D (1976) Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* **15**: 3-24
- Evans IM, Gatehouse JA, Boulter D (1985) Regulation of storage-protein synthesis in pea (*Pisum sativum* L.) cotyledons under conditions of sulphur deficiency. *Biochem J* **232**: 261-265
- Finkelstein RR, Somerville CR (1990) Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. *Plant Physiol* **94**: 1172-1179
- Finkelstein RR, Tenbarger KM, Shumway JE, Crouch ML (1985) Role of ABA in maturation of rapeseed embryos. *Plant Physiol* **78**: 630-636

- Fujiwara T, Hirai MY, Chino M, Komeda Y, Naito S (1992) Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. *Plant Physiol* **99**: 263–268
- Gayler KR, Sykes GE (1981) β -Conglycinins in developing soybean seeds. *Plant Physiol* **67**: 958–961
- Gayler KR, Sykes GE (1985) Effects of nutritional stress on the storage proteins of soybeans. *Plant Physiol* **78**: 582–585
- Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *Plant Cell* **4**: 1251–1261
- Guerche P, Tire C, Grossi de Sa F, De Clercq A, Van Montagu M, Krebbers E (1990) Differential expression of the *Arabidopsis* 2S albumin genes and the effect of increasing gene family size. *Plant Cell* **2**: 469–478
- Heath JD, Weldon R, Monnot C, Meinke DW (1986) Analysis of storage proteins in normal and aborted seeds from embryo-lethal mutants of *Arabidopsis thaliana*. *Planta* **169**: 304–312
- Holowach LP, Madison JT, Thompson JF (1986) Studies on the mechanism of regulation of the mRNA level for a soybean storage protein subunit by exogenous L-methionine. *Plant Physiol* **80**: 561–567
- Holowach LP, Thompson JF, Madison JT (1984) Effects of exogenous methionine on storage protein composition of soybean cotyledons cultured *in vitro*. *Plant Physiol* **74**: 576–583
- Hughes DW, Galau GA (1991) Developmental and environmental induction of *Lea* and *LeaA* mRNAs and the postabscission program during embryo culture. *Plant Cell* **3**: 605–618
- Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiol* **90**: 463–469
- Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. *Theor Appl Genet* **61**: 385–393
- Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol Plant* **61**: 377–383
- Krebbers E, Herdies L, De Clercq A, Seurinck J, Leemans J, Van Damme J, Segura M, Gheysen G, Van Montagu M, Vandekerckhove J (1988) Determination of the processing sites of an *Arabidopsis* 2S albumin and characterization of the complete gene family. *Plant Physiol* **87**: 859–866
- Ladin BF, Tierney ML, Meinke DW, Hosangadi P, Veith M, Beachy RN (1987) Developmental regulation of β -conglycinin in soybean axes and cotyledons. *Plant Physiol* **84**: 35–41
- Leutwiler LS, Hough-Evans BR, Meyerowitz EM (1984) The DNA of *Arabidopsis thaliana*. *Mol Gen Genet* **194**: 15–23
- Lloyd AM, Barnason AR, Rogers SG, Byrne MC, Fraley RT, Horsch RB (1986) Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. *Science* **234**: 464–466
- Meinke DW, Chen J, Beachy RN (1981) Expression of storage protein genes during soybean seed development. *Planta* **153**: 130–139
- Meyerowitz EM, Pruitt RE (1985) *Arabidopsis thaliana* and plant molecular genetics. *Science* **229**: 1214–1218
- Naito S, Dubè PH, Beachy RN (1988) Differential expression of conglycinin α and β subunit genes in transgenic plants. *Plant Mol Biol* **11**: 109–123
- Nambara E, Naito S, McCourt P (1992) A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. *Plant J* **2**: 435–441
- Pang PP, Pruitt RE, Meyerowitz EM (1988) Molecular cloning, genomic organization, expression and evolution of 12S seed storage protein genes of *Arabidopsis thaliana*. *Plant Mol Biol* **11**: 805–820
- Randall PJ, Thompson JA, Schroeder HE (1979) Cotyledonary storage proteins in *Pisum sativum*. IV. Effects of sulfur, phosphorus, potassium and magnesium deficiencies. *Aust J Plant Physiol* **6**: 11–24
- Rogers SG, Klee HJ, Horsch RB, Fraley RT (1987) Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *Methods Enzymol* **153**: 253–277
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning*. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Shewry PR, Franklin J, Parmar S, Smith SJ, Mifflin BJ (1983) The effects of sulphur starvation on the amino acid and protein composition of barley grain. *J Cereal Sci* **1**: 21–31
- Spencer D, Rerie WG, Randall PJ, Higgins TJV (1990) The regulation of pea seed storage protein genes by sulfur stress. *Aust J Plant Physiol* **17**: 355–363
- Tierney ML, Bray EA, Allen RD, Ma Y, Drong RF, Slightom J, Beachy RN (1987) Isolation and characterization of a genomic clone encoding the β -subunit of β -conglycinin. *Planta* **172**: 356–363
- Wrigley CW, Cros DL, Fullington JG, Kasarada DD (1984) Changes in polypeptide composition and grain quality due to sulfur deficiency in wheat. *J Cereal Sci* **2**: 15–24