Effects of Sulfur Nutrition on Expression of the Soybean Seed Storage Protein Genes in Transgenic Petunia[1]

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ABSTRACT

The 7S seed storage protein (β-conglycinin) of soybean (*Glycine max* [L]. Merr.) has three major subunits; α, α', and β. Accumulation of the β-subunit, but not the α- and α'-subunits, has been shown to be repressed by exogenously applied methionine to the immature cotyledon culture system (LP Holowach, JF Thompson, JT Madison [1984] Plant Physiol 74: 576–583) and to be enhanced under sulfate deficiency in soybean plants (KR Gayler, GE Sykes [1985] Plant Physiol 78: 582–585). Transgenic petunia (*Petunia hybrida*) harboring either the α'- or β-subunit gene were constructed to test whether the patterns of differential expression were retained in petunia. Petunia regulates these genes in a similar way as soybean in response to sulfur nutritional stimuli, *i.e.* (a) expression of the β-subunit gene is repressed by exogenous methionine in *in vitro* cultured seeds, whereas the α'-subunit gene expression is not affected; and (b) accumulation of the β-subunit is enhanced by sulfur deficiency. The pattern of accumulation of major seed storage protein of petunia was not affected by these treatments. These results indicate that this mechanism of gene regulation in response to sulfur nutrition is conserved in petunia even though it is not used to regulate its own major seed storage proteins.

Soybean is one of the major protein sources for human and agriculturally important animals. However, the amino acid composition of seed protein is not ideal because of its poor content of sulfur-containing amino acids. Soybean seeds contain two major seed storage protein components, namely, 11S protein (glycinin) and 7S protein (β-conglycinin). Glycinin, which consists of approximately 50% of total seed protein, is relatively rich in sulfur-containing amino acids (1.8%). β-Conglycinin, the second abundant protein, is relatively poor in these amino acids (0.6%) (see ref. 28 for review). β-Conglycinin forms homo- or heterotrimers of three subunits, namely, α' (76 kD), α (72 kD), and β (53 kD). Among these, the β-subunit has an especially low sulfur content (10). By increasing the content of sulfur rich protein and decreasing the levels of sulfur-poor proteins, one can improve the nutritional quality of soybean.

Expression of the β-conglycinin genes has been studied extensively. All three subunit genes of the β-conglycinin are expressed specifically in middle to late stages of soybean seed development, although expression of each subunit gene is regulated differently in terms of timing and tissue specificity of expression (16, 22, 24, 25). The levels of expression are also different. Copy numbers of the α'- and β-subunit genes are estimated to be at least three (18) and eight to 13 (30) per haploid genome, respectively, whereas the levels of each protein accumulated in soybean seeds are approximately the same (16), indicating that the α'-subunit gene has a higher expression level than the β-subunit gene per gene copy. When these genes were introduced into transgenic tobacco or petunia plants, the pattern of expression was found to be similar to that in soybean plants in terms of the timing (5, 8, 9, 25), levels of accumulation (25), and tissue specificity (2, 5, 8, 9). These facts indicate that tobacco, petunia, and soybean have a common mechanism of seed-specific gene regulation.

These genes are also known to respond differently to nutritional stimuli. Holowach *et al.* (21) reported that, in their *in vitro* culture system of immature soybean cotyledons, accumulation of the β-subunit protein was completely repressed when cotyledons were cultured in the presence of methionine, whereas the accumulation of the α- and α'-subunits were little affected. The suppression of the β-subunit accumulation is regulated at the mRNA level (11) and the repression is reversible, *i.e.* accumulation of mRNA coding for the β-subunit reinitiates after removal of methionine from the medium (20). Sulfur or potassium deficiency also affects the seed storage protein composition of soybean (17). Under sulfur deficiency, more β-conglycinin accumulates than glycinins. Subunit composition of β-conglycinin is also affected, and there is a threefold increase in the accumulation of the β-subunit protein, whereas the other two subunits are little affected.

To understand the molecular mechanism(s) of nutritional regulation of soybean seed storage protein gene expression, we attempted to build a system in which artificially introduced β-conglycinin genes would respond to nutritional stimuli in a similar way as in soybean plants. Transient expression after direct gene transfer and stable transformation could be used to accomplish this purpose. We chose to build transgenic petunia harboring β-conglycinin genes using *Agrobacterium*-mediated gene transfer because it has been shown that the β-conglycinin genes were expressed when introduced into protoplasts from various sources of nonembryonic origin and did not show methionine or ABA responses (15). In this paper,
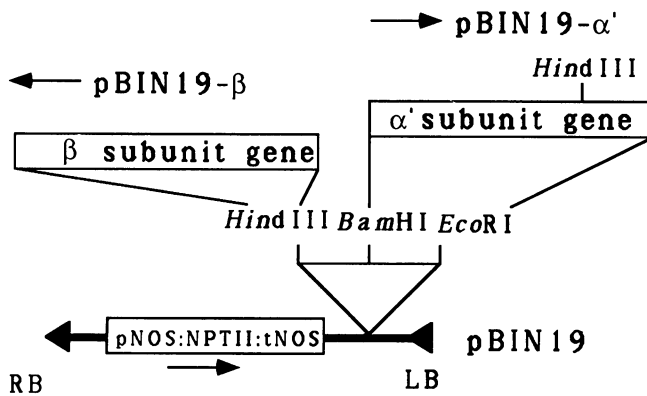
Figure 1. Plasmid maps. All steps of cloning are described in the text. Transferred-DNAs of the final plasmids are shown. A single *Hin*dIII site is present in the coding region of the α'-subunit gene (2.8 kb from *Bam*HI site). Arrows indicate directions of transcription. LB, left border; RB, right border; pNOS, nopaline synthase gene promoter; NPTII, neomycin phosphotransferase II gene; tNOS, nopaline synthase gene 3' end.

we report that the accumulation of the β-subunit protein in transgenic petunia plants responds to sulfur nutritional stimuli in a similar way to that of soybean.

MATERIALS AND METHODS

Plasmid Construction

All procedures used for DNA manipulation were carried out according to the standard procedures of Maniatis *et al.* (23). A 3.8-kb[2] DNA fragment coding for the α'-subunit gene was isolated from the Gmg17.1-Δ904 clone (9, kindly provided by Dr. R.N. Beachy) by digestion with *Bam*HI and *Eco*RI and was recloned into the corresponding sites of pBIN19 (6) to yield pBIN19-α' (Fig. 1). The α'-gene contains 0.9 and 0.3 kb of its 5'- and 3'-flanking sequences, respectively, which have been shown to be sufficient to maintain a high level of expression in both a tissue-specific and temporal-specific manner in transgenic plants (9). A 4.2-kb DNA fragment coding for the β-subunit gene was excised from pGmg91 (30; also provided by Dr. R.N. Beachy) by digestion with *Hin*dIII and recloned into the same site of pBIN19 to yield pBIN19-β. The orientation of the β-subunit gene in pBIN19-β is opposite to that of the α'-subunit gene in pBIN19-α' (Fig. 1). The β-subunit gene has 1.1 and 1.2 kb, respectively, of its 5' and 3' regions. The tissue specificity and temporal pattern of expression of this gene in transgenic plants have been shown to be similar to those in soybean (8).

[2] Abbreviations: kb, kilobase; MGRL media, 1.75 mM sodium phosphate buffer (pH 5.8), 1.5 mM $MgSO_4$, 2.0 mM $Ca(NO_3)_2$, 3.0 mM KNO_3, 67 μM Na_2EDTA, 8.6 μM $FeSO_4$, 10.3 μM $MnSO_4$, 30 μM H_3BO_3, 1.0 μM $ZnSO_4$, 24 nM $(NH_4)_6Mo_7O_{24}$, 130 nM $CoCl_2$, 1 μM $CuSO_4$.

Construction of Transgenic Petunia Carrying the α'- or β-Subunit Gene

pBIN19-α' and pBIN19-β, as well as pBIN19, were introduced into *Agrobacterium tumefaciens* strain C58C1 Rif[R] (pGV2260) (12) by triparental mating using pRK2013 (14) as a helper. The resulting *A. tumefaciens* strains were used to infect petunia (*Petunia hybrida* cv VR hybrid) leaf discs. Transgenic plants were regenerated within 3 months of *Agrobacterium* infection according to the method of Naito *et al.* (25). Total DNA was isolated from leaves of each transgenic plant and subjected to Southern analysis. DNA extraction and Southern analysis were performed according to the method of Naito *et al.* (25) except that fractionated DNA was transferred onto nylon membranes (Gene Screen Plus, New England Nuclear, Boston, MA). Random priming was used for radiolabeling the probes. Seed protein was extracted and the α'- and β-subunit proteins were detected by western blotting using rabbit anti-soybean 7S antibody (kindly provided by Dr. R.N. Beachy) by the procedure of Naito *et al.* (25). In most cases, two SDS-PAGE analyses were run in parallel, one was subjected to western analysis and the other was stained with Coomassie brilliant blue R250.

Petunia Pod Culture

Transgenic petunia plants were grown under fluorescent light at 800 μE m^{-2} s^{-1} with a 16-h light/8-h dark photoperiod in a mixture of perlite:vermiculite:peat moss (1:1:1) at 22°C. MGRL media was used to water plants. Flowers were hand-pollinated and tagged on the days of flowering. Immature seed pods of appropriate maturing stages were cut at the petioles and surface sterilized in a 10-fold dilution of commercial bleach solution containing 0.01% (v/v) Triton X-100 with gentle agitation for 15 min followed by several rinses with sterile water. After the sepals were removed from the surface-sterilized pods, the pods were cut in half longitudinally along the joint of the two carpels, then put on agar plates with the cut surface facing the agar, and cultured under the same temperature and light conditions as for the transgenic plants. The culture media were essentially the same as the basal and methionine-supplemented media described by Holowach *et al.* (21) except that the media were solidified by 1% agar (Shoei, Tokyo, Japan). One-half of each seed pod was cultured on the basal medium, and the other half was cultured on the methionine-supplemented (8.4 mM) medium. The period of culture was 6 d for protein analysis and 4 d for mRNA analysis. After each period of culture, seeds were collected and immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA extraction and northern analysis were done as described by Naito *et al.* (25) except that nylon membranes (Gene Screen Plus) were used. Protein extraction and its western analysis were also done according to the method of Naito *et al.* (25).

Hydroponic Culture

Cuttings were made from the original transformant line TFPhβ-1 and were cultured in control or sulfur-deficient hydroponic solution under continuous white fluorescent light

at 400 μE m^{-2} s^{-1} and 22°C. The control hydroponic culture medium was the MGRL medium. The sulfate-deficient hydroponic culture medium had the same composition as the MGRL medium except that MgSO$_4$ was replaced by an equal molar concentration of MgCl$_2$. Sulfate concentrations of the control and sulfate-deficient media were 1.5 mM and approximately 20 μM, respectively. Mature seeds from these cuttings were subjected to protein analysis as described above.

RESULTS

### Construction of Transgenic Petunia Carrying the α'- or β-Subunit Gene

Several independent transgenic plants carrying each construct were recovered. Plant lines TFPhβ-1, 2, 8, 13 were recovered from transformation with pBIN19-β, TFPhα'-1, 2, 14, 16 with pBIN19-α', and TFPhBIN-2, 5, 7 with pBIN19. Total DNA was extracted from leaves of each plant line and was analyzed by Southern hybridization after digestion with HindIII (Fig. 2A). All TFPhβ lines showed a 4.2-kb band that corresponds to the intact β-subunit gene. For transformation with pBIN19-β, the copy number of the β-gene inserted was $\leq$10 copies per diploid genome. Among them, TFPhβ-8 seemed to be a single-copy transformant. On the other hand, all TFPhα' lines contained many copies of the trans-gene. All TFPhα' lines showed an intense 2.8-kb band in addition to several high mol wt bands. The 2.8-kb bands correspond to the internal HindIII fragment containing the α'-subunit gene. The 3.6-kb band seen in the copy number control lanes

corresponds to pUC9 (vector of Gmg17.1-Δ904 clone) plus the 3' end of the α'-subunit gene (1.0 kb). The number of high mol wt bands represents the number of different loci where gene insertion occurred because there is no HindIII site between the internal HindIII site of the α'-subunit gene and the left border. Wild-type petunia DNA did not hybridize to the probe (data not shown). Judging from the intensity of the 2.8-kb bands and the numbers of high mol wt bands, >10 copies were introduced to all TFPhα' lines in at least several different loci.

Protein was extracted from mature seeds of each line (primary transgenic plants) and expression of the introduced genes was analyzed by western blotting. As shown in Figure 2B, transgenic petunia carrying the β-conglycinin gene accumulated protein(s) that interact(s) with anti-soybean 7S antiserum, whereas none of the TFPhBIN lines had detectable protein. In the case of TFPhβ lines, the protein had the same mobility as the β-subunit in mature soybean seed (53 kD). However, in the case of TFPhα' lines, proteins recognized by anti-soybean 7S antiserum had various sizes ranging from 55 kD up to the size of the α'-subunit protein in soybean seed (76 kD). These results are consistent with previous reports (9, 25), indicating that the α'-subunit protein is susceptible to proteolytic cleavage in transgenic petunia and tobacco seeds in which the most abundant breakdown product was 55 kD.

Judging from intensities of bands, copy numbers of trans-gene and levels of the α'- or β-subunit accumulation are correlated well. β-Conglycinin genes have been reported to have little chromosomal position effect (8, 9, 25). The results

Figure 2. Characterization of transgenic plants. A, Determination of copy number by Southern hybridization. Total DNA from transgenic petunia was digested by HindIII and subjected to 0.7% agarose gel electrophoresis. Fractionated DNA was transferred to a nylon membrane and probed by ^{32}P-labeled α' (BamHI-EcoRI fragment for TFPhα') or β (HindIII fragment for TFPhβ) subunit gene fragment. Appropriate amounts of HindIII digests of plasmid DNA (Gmg17.1-Δ904 in pUC9 for TFPhα' lines and pGmg91 in pSP64 for TFPhβ lines) were used to constitute copy number controls. W, Untransformed petunia. B, Detection of β-conglycinin in transgenic petunia seeds by western blotting. Seed protein was extracted as described in the text, and 50 μg of total protein was subjected to 12.5% SDS-PAGE, followed by western blotting. The subunits of β-conglycinin were then detected by rabbit anti-7S antiserum. The 55- and 53-kD bands correspond to the α'- and β-subunit, respectively. Total soybean (cv Toyosuzu) seed extract (0.5 and 2 μg of total protein) was used as standard (SB lanes). W, Untransformed petunia.
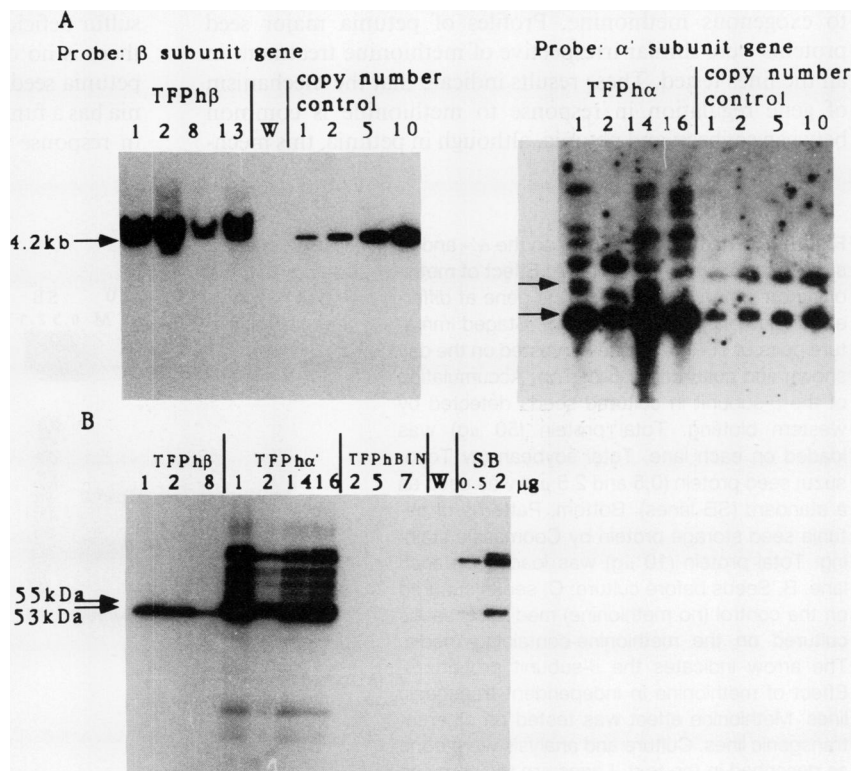
obtained here are consistent with these observations, even though the vector used for transformation of petunia was different from that used in the previous studies. This indicates that the β-conglycinin genes themselves behave in a chromosomal position-independent manner.

Response of α'- and β-Subunit Gene Expression in *in Vitro* Cultured Pods to Exogenous Methionine

Immature seed pods from TFPhβ-1 at various stages (12, 16, and 20 DAF) were cut longitudinally into halves and were cultured on solid media with or without methionine for 6 d. Protein extracts were analyzed by western blotting. As shown in Figure 3A, seeds cultured on the basal medium contained more β-subunit protein than did seeds before culture, indicating that the β-subunit protein accumulated during the culture period. Profiles of petunia major storage proteins were relatively unchanged during the culture. Irrespective of the stages of seed development, the accumulation of the β-subunit protein during culture was repressed when the immature seed pods were cultured in the presence of methionine. Methionine represses the β-subunit gene expression in petunia even at later stages of development when the expression has already initiated. This fact is consistent with the results obtained in soybean, in which the repression has been shown to be independent of the developmental stage (20). The repression was most evident when pods at 16 DAF were used for culture. Based on this result, subsequent cultures were started at 16 DAF.

Two other transgenic lines carrying the β-subunit gene (TFPhβ-8 and TFPhβ-13) showed a similar response (Fig. 3B). In contrast, TFPhα'-1 and TFPhα'-14 did not respond to exogenous methionine. Profiles of petunia major seed proteins were similar irrespective of methionine treatment in all the lines tested. These results indicate that the mechanism of gene regulation in response to methionine is common between soybean and petunia, although in petunia, this mech-

anism is evidently not used to regulate its endogenous major storage protein accumulation.

In the case of soybean, it has been demonstrated that the effect of methionine is at the mRNA level (11, 20). Total RNA from 4-d-cultured immature seeds of TFPhβ-1 were subjected to northern analysis to determine how methionine affects mRNA accumulation (Fig. 4). Seeds cultured on the control media had a higher level of the β-subunit mRNA accumulation than seeds before culture, indicating that expression of the β-subunit message occurred during culture. Much less β-subunit mRNA accumulated in immature seeds treated with methionine than in seeds cultured without methionine.

These results indicate that the effect of methionine on the expression of the β-subunit gene in petunia is similar to that seen in soybean, in that accumulation of the α'-subunit protein is not affected, whereas accumulation of β-subunit protein as well as level of mRNA coding for the β-subunit is reduced.

Response of the β-Subunit Gene to Sulfur Deficiency

Cuttings from TFPhβ-1 were cultured in the control or sulfur-deficient hydroponic solutions to see the effect of sulfur deficiency on expression of the β-subunit gene in petunia. TFPhβ-1 plants cultured under sulfur deficiency were smaller in size and developed fewer leaves than those cultured on control media. Root mass of sulfur-deficient TFPhβ-1, however, developed much better than control (data not shown). Proteins from mature seeds were subjected to western analysis. As shown in Figure 5, TFPhβ-1 accumulated severalfold more β-subunit protein in seeds when the plants were grown under sulfur deficiency. Coomassie blue staining patterns show that there is no obvious difference in the accumulation of major petunia seed storage proteins. This again indicates that petunia has a functional mechanism to regulate the β-subunit gene in response to sulfur deficiency and that the mechanism is

Figure 3. Effect of methionine on the α'- and β-subunit protein accumulation. A, Effect of methionine on expression of β-subunit gene at different stages of seed development. Staged immature pods of TFPhβ-1 were harvested on the day shown and cultured for 6 d. Top, Accumulation of the β-subunit in cultured seeds detected by western blotting. Total protein (50 μg) was loaded on each lane. Total soybean (cv Toyosuzu) seed protein (0.5 and 2.5 μg) was used as a standard (SB lanes). Bottom, Patterns of petunia seed storage protein by Coomassie staining. Total protein (10 μg) was loaded on each lane. B, Seeds before culture; C, seeds cultured on the control (no methionine) media; M, seeds cultured on the methionine-containing media. The arrow indicates the β-subunit protein. B, Effect of methionine in independent transgenic lines. Methionine effect was tested on different transgenic lines. Culture and analysis were done as described in the text. Lanes are the same as in A.
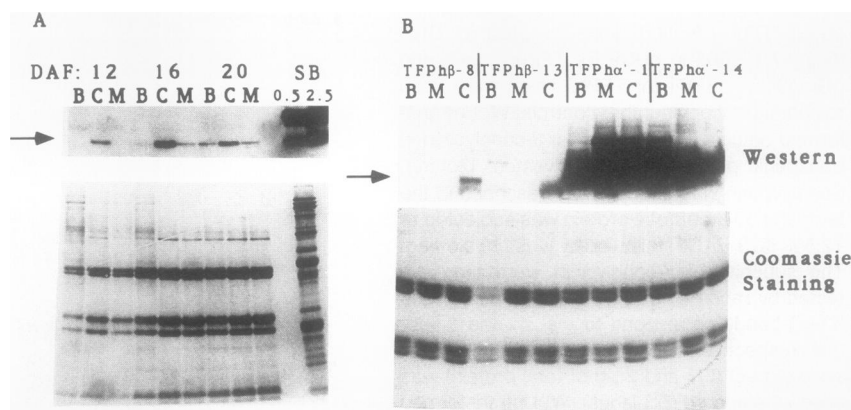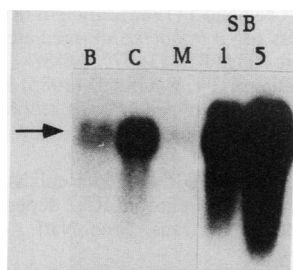
Figure 4. Effect of methionine on the β-subunit mRNA accumulation. Total RNA was extracted from 4-d-cultured (starting at 16 DAP) seeds of TFPhβ-1, and 5 μg of RNA was subjected to electrophoresis. Fractionated RNA was blotted onto a nylon membrane and probed by ³²P-labeled β-subunit gene fragment. B, Seeds before culture; C, seeds cultured on control (no methionine) media; M, seeds cultured on methionine-containing media. Total RNA isolated from immature soybean (cv Provar) cotyledon (1 and 5 μg) was used as a standard (SB lanes). The arrow indicates the position of the β-subunit mRNA.

apparently not utilized to regulate its own major seed protein genes.

DISCUSSION

Studies of plant responses to nutritional stimuli are important because adequate nutrition is essential for proper growth of plants. The understanding of plant nutrition has a great potential for agricultural application. For this reason, an enormous amount of research has been conducted mainly by physiological approaches. Changes in nutritional condition cause a wide variety of physiological responses as well as changes in patterns of gene expression. Mainly because of the complex nature of responses, it is still not very clear how changes in nutritional condition are perceived and how signals are transmitted and transduced in plants.

The effect of sulfur nutrition on accumulation of seed storage proteins is one of the ideal models to reveal the unknown links between plant nutrition and gene expression. Sulfur deficiency increases the level of sulfur-poor proteins in many plants including pea (26), lupin (7), wheat (33), barley (27), maize (3), cowpea (13), rape, and sunflower (29) in addition to soybean. Under limited sulfur availability, plants maintain overall levels of seed storage protein by accumulating more storage proteins of low content of sulfur-containing amino acids and less of those with high content (19). In the case of pea, it has been shown that changes in the accumulation of seed proteins in response to sulfur deficiency are regulated at both the transcriptional and posttranscriptional levels (4). The fact that similar responses have been observed among a great diversity of plant species that have different sets of storage proteins suggests that this response is universal and may be essential. On the other hand, little is known concerning methionine regulation of storage proteins among various plant species. Soybean is the only example described so far.

In this report, we demonstrate that expression of the β-subunit of β-conglycinin in transgenic petunia is repressed by exogenous methionine in in vitro pod culture, whereas it has no effect on accumulation of the petunia major storage pro-

teins and the α'-subunit, which does not respond to methionine in soybean. This indicates that petunia has a functional mechanism to regulate soybean storage protein synthesis in response to exogenous methionine in a way similar to that of soybean, although it seems that petunia does not use this mechanism to regulate its own major seed protein synthesis. The effect of exogenously applied methionine has been shown to be at the level of mRNA accumulation in soybean. In our in vitro culture of transgenic petunia pods, addition of methionine repressed accumulation of the β-subunit mRNA, indicating that the mechanism is similar in soybean and in petunia. It has been shown that levels of free methionine remain relatively constant in a wide range of sulfur availability (see ref. 1 for review). The methionine content of seed declines during seed development of soybean, which may be responsible for the late initiation of the β-subunit accumulation (31). Our observations may suggest that methionine content of petunia seeds also declines in the course of seed maturation and this is the cause of the difference of timing of expression of the α'- and β-subunit gene in transgenic petunia seeds (25).

We also showed that petunia has a mechanism to control the accumulation of soybean seed storage protein in response to sulfur deficiency. The fact that the trans-gene is regulated in a similar way as it is in soybean indicates that not only do plants show similar responses to sulfur deficiency but also the mechanism of regulation of seed storage protein composition is common. Again, petunia does not seem to use this mechanism to control its own major seed storage protein synthesis. Spencer et al. (29) showed that pea legumin, which is known to be down-regulated by sulfur deficiency in pea, accumulated to a reduced level under sulfur deficiency in transgenic tobacco seeds, whereas no change is observed in the tobacco
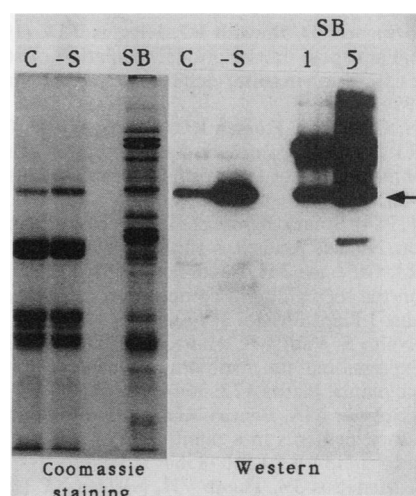
Figure 5. Effect of sulfur deficiency on accumulation of the β-subunit protein in transgenic petunia seeds. Total protein was extracted from mature seeds of TFPhβ-1 cultured under either control medium (C) or sulfate-deficient medium (−S) and analyzed by western blotting (right). Total petunia protein (50 μg) was loaded except for total soybean (cv Toyosuzu) seed extract (1 and 5 μg, SB lanes). Left, Coomassie staining pattern of petunia seed protein. Total protein (10 μg) was loaded onto each lane. The arrow indicates the position of the β-subunit protein.

storage protein accumulation. These facts indicate that plants share mechanisms of both up-regulation and down-regulation of storage protein synthesis in response to sulfur deficiency and that these mechanisms are present even in species that do not have storage protein genes to be regulated by these mechanisms.

It is widely accepted that dicot plants have common mechanisms of gene regulation. Most of the dicot genes put stably in heterologous dicot hosts retain their patterns of expression, i.e. organ specificity, temporal pattern of expression, and inducibility by environmental stimuli (see ref. 32 for review). Our findings indicate that plants also share common mechanisms of gene regulation in response to sulfur nutritional stimuli. This allows the identification of cis-acting element(s) in the genes responsible for these responses using transgenic plants. These experiments will help us to understand the molecular mechanism(s) of gene regulation in response to nutritional stimuli, which is one of the most important aspects of plant nutrition.

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