Genetic Modification Removes an Immunodominant Allergen from Soybean[1][w]

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The increasing use of soybean (*Glycine max*) products in processed foods poses a potential threat to soybean-sensitive food-allergic individuals. In vitro assays on soybean seed proteins with sera from soybean-sensitive individuals have immunoglobulin E reactivity to abundant storage proteins and a few less-abundant seed proteins. One of these low abundance proteins, Gly m Bd 30 K, also referred to as P34, is in fact a major (i.e. immunodominant) soybean allergen. Although a member of the papain protease superfamily, Gly m Bd 30 K has a glycine in the conserved catalytic cysteine position found in all other cysteine proteases. Transgene-induced gene silencing was used to prevent the accumulation of Gly m Bd 30 K protein in soybean seeds. The Gly m Bd 30 K-silenced plants and their seeds lacked any compositional, developmental, structural, or ultrastructural phenotypic differences when compared with control plants. Proteomic analysis of extracts from transgenic seed detected the suppression of Gly m Bd 30 K-related peptides but no other significant changes in polypeptide pattern. The lack of a collateral alteration of any other seed protein in the Gly m Bd 30 K-silenced seeds supports the presumption that the protein does not have a role in seed protein processing and maturation. These data provide evidence for substantial equivalence of composition of transgenic and non-transgenic seed eliminating one of the dominant allergens of soybean seeds.

Food allergy can be a serious nutritional problem in children and adults, and any food that contains protein has the potential to elicit an allergic reaction in a percentage of the human population. Avoidance of the food is the only treatment available, thus severely limiting dietary choices and the quality of life of food-allergic individuals. More than 90% of all food allergies in the United States are attributable to cows' milk, eggs, fish, crustaceans, peanuts, soybeans (*Glycine max*), tree nuts, and wheat (*Triticum aestivum*; Taylor and Hefle, 2001). The allergens in foods are almost always naturally occurring proteins. Although foods contain millions of individual proteins, only a comparative few food proteins have been documented as being allergens. Some foods are known to contain multiple allergenic proteins, including soybeans, peanuts, cows' milk, and eggs (Thanh and Shibasaki, 1976; Nordlee et al., 1981; Burks et al., 1988; Bush and Hefle, 1996; Taylor and Hefle, 2001).

Increased awareness of the many health benefits of soy protein, along with improved isolation techniques resulting in better flavor and increased functionality, has resulted in widespread use of soy protein isolates and concentrates in a variety of food products. Although this is of benefit to the general population, it is becoming increasingly difficult for sensitive individuals to avoid soy products in prepared and processed foods (Herian et al., 1990; Vidal et al., 1997).

Three soybean proteins, Gly m Bd 60 K, Gly m Bd 30 K, and Gly m Bd 28 K represent the main seed allergens in soybean-sensitive patients (Ogawa et al., 2000). Many soy-sensitive patients will react to only one protein, although some, especially those with cross-reactivity to peanuts, will react to multiple proteins (Herian et al., 1990). In a number of IgE binding studies, it has been shown that more than 65% of soy-sensitive patients react only to the Gly m Bd 30 K protein (Ogawa et al., 1993, 1991; Helm et al., 1998, 2000). Thus, even though it is a relatively minor seed constituent (less than 1% of total seed protein), Gly m Bd 30 K is regarded as the major or immunodominant soybean allergen.

The molecular identity of the genes encoding these protein allergens has been revealed in recent years. Gly m Bd 60 K is the α-subunit of β-conglycinin, one of the very abundant soybean seed storage proteins (Ogawa et al., 1995); Gly m Bd 28 K is an MP27-MP33 homolog, a minor soybean seed globulin (Tsuji et al.,

1995). The other, Gly m Bd 30 K, has been shown to be identical to the previously described soybean seed 34-kD seed vacuolar protein, P34 (Kalinski et al., 1990, 1992; Ogawa et al., 1993). This protein is a member of the papain superfamily of Cys proteases (Kalinski et al., 1990, 1992). Like other members of this family, it is initially synthesized as a larger precursor and is posttranslationally processed. The function of Gly m Bd 30 K is not clear, and no enzymatic activity has been reported. It differs from all other described papain-type proteases by possessing a Gly substitution for a conserved Cys in the active site.

It has been possible to reduce or remove some of these allergens from soybean by development of mutant lines (Samoto et al., 1997; Ogawa et al., 2000). However, mutagenesis and breeding have not been successful for the dominant allergen Gly m Bd 30 K, which can only be removed from soy protein fractions by specialized processing techniques that limit the use of the resultant protein isolate (Samoto et al., 1997; Ogawa et al., 2000). During the industrial fractionation of soybean proteins, it remains in part associated with the seed globulins that are the major constituents of soybean protein isolates. It is consequently present in most processed food products that contain soybean protein (Ogawa et al., 1993, 200).

Biotechnology offers the prospect of using recombinant techniques to eliminate undesirable proteins such as Gly m Bd 30 K to enhance food safety and possibly make the use of soybean products available to many sensitive individuals. In this paper, we describe the creation of genetically modified soybean lines by transgene-induced gene silencing of the Gly m Bd 30 K gene. The resultant transgenic seeds do not accumulate the Gly m Bd 30 K protein and appear to be otherwise unchanged by the removal of this allergenic protein.

RESULTS

Isolation of Gly m Bd 30 K Null Lines and Regeneration of Plants

Immunological analysis of embryos regenerated from embryogenic suspension cells initially confirmed that the Gly m Bd 30 K protein is accumulated in somatic embryos (data not shown). This permitted us to develop an immunoblot-based assay to evaluate silencing of Gly m Bd 30 K protein expression in lines of transgenic somatic embryos.

The silencing of the endogenous Gly m Bd 30 K gene was accomplished by transforming soybean somatic embryos with a DNA construct containing the full open reading frame (ORF) of a Gly m Bd 30 K cDNA. This was under the control of a β-conglycinin promoter with a phaseolin termination region as described in "Materials and Methods." Transgenic events resistant to the selectable marker hygromycin were clonally propagated and screened for presence or absence of Gly m Bd 30 K protein. Among these

lines, a candidate was identified that exhibited apparent Gly m Bd 30 K suppression, and this line was regenerated into six R0 plants.

Western blotting, using the Gly m Bd 30 K monoclonal antibody, of protein extracts from seed chips, showed the seeds (R1) of these self-fertilized, R0 plants were segregating for the Gly m Bd 30 K-silenced phenotype. The remaining R1 seeds with Gly m Bd 30 K suppression were propagated a further generation to obtain homozygous plants. Seeds from these R0 plants with a wild-type phenotype were also propagated as controls. The seeds of R2 plants and their progeny (R3 seeds) were also assayed immunologically using the Gly m Bd 30 K monoclonal antibody. The plants showed dominant, Mendelian inheritance and stability of the trait for these three generations. The plants matured, flowered, and set seed in the same developmental pattern as the wild-type controls. More importantly, the seed sizes, shapes, protein- and oil contents were also indistinguishable from the controls (not shown). The proteomic analysis described below was conducted on homozygous R3 seeds.

Immunological Data Indicated Complete Suppression of Gly m Bd 30 K Protein

Immunoblots of seed proteins were probed with either with a monoclonal Gly m Bd 30 K antibodies (Fig. 1, panel 2) or with human IgE (Fig. 1, panel 3) contained in a pooled serum of six soybean-sensitive people (Helm et al., 2000). Separated in the first lane was protein extracted from seed of a transgenic line lacking the α- and α'-subunits of β-conglycinin (Fad
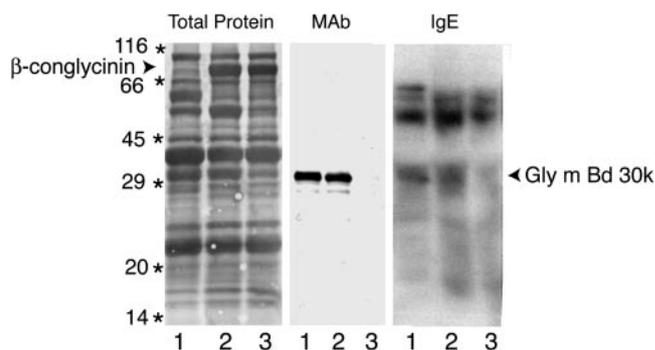

Figure 1. Removal of Gly m Bd 30 K protein as a consequence of gene silencing. The three panels show replicate samples stained for total protein with amido black and labeled either with anti-Gly m Bd 30 K monoclonal antibody or with a mixture of IgEs from six soybean-sensitive individuals. Lane 1, Transgenic control of another soybean suppressing the α/α'-conglycinin (arrowhead) that does contain Gly m Bd 30 K (Kinney et al., 2001). Lane 2, Wild-type soybean cv Jack control. Lane 3, R3 generation homozygous transgenic soybean with the Gly m Bd 30 K construct inducing the complete suppression of Gly m Bd 30 K accumulation. The lanes in all the gels were equally loaded with 200 μg of seed protein extracts from the three soybean lines. The M_r indications correspond to M_r markers ran in additional lanes.

2, G19) as previously described (Kinney et al., 2001). The two other lanes in the gel contained protein extracted from the lines with an ectopic copy of the Gly m Bd 30 K transgene and the other a wild-type control (soybean cv Jack). The protein profiles in these two lanes appeared indistinguishable from each other (Fig. 1, lanes 2 and 3). The equally loaded protein gel lanes exhibited different patterns when analyzed for Gly m Bd 30 K immunoreactive proteins (Fig. 1, panel 2) and proteins immunoreactive with IgEs from soybean-sensitive people (Fig. 1, panel 3). The first two protein samples had a strongly immunoreactive polypeptide migrating with a relative mass of 30 kD, and in contrast, this signal was completely missing from the third sample (Fig. 1, panel 3). This result is consistent with the presence of Gly m Bd 30 K in the controls and the silencing of Gly m Bd 30 K expression in the transgenic line, as expected. The IgE immunoblots showed a more complex pattern of signals because the IgE in pooled human sera cross-react with several immunodominant polypeptides (Fig. 1, panel 3). However, Gly m Bd 30 K accounted for one of the two major IgE immunoreactive signals (Fig. 1, panel 3, lanes 1 and 2). The other major cross-reactive band was identified as Gly m Bd 50 K α/α' β-conglycinin (Fig. 1, panel 3, lanes 2 and 3), which was absent in extracts from seed of the line suppressed in α/α' β-conglycinin, as expected. The IgE immunoreactivity for the Gly m Bd 30 K polypeptide was absent in Gly m Bd 30 K-suppressed seed protein extracts, and it is also important that none of the other immunoreactive signals appeared to be increased in this seed protein extract.

Protein Storage Vacuoles in Gly m Bd 30 K-Suppressed Plants Are Indistinguishable from the Control

Electron microscopic-immunocytochemical assays were conducted to test whether there were any observable subcellular structural differences between the Gly m Bd 30 K-silenced line and control plants accumulating Gly m Bd 30 K. The protein storage vacuoles in both the Gly m Bd 30 K-containing controls and the Gly m Bd 30 K-silenced line appeared to be identical in morphology (Fig. 2, A and B). Immunogold assays (Fig. 2, A and B) confirmed that Gly m Bd 30 K signals were absent in the protein storage vacuole of the suppressor line and were present in control line.

A Comparative Two-Dimensional Gel/Mass Spectrometric (MS) Proteome Analysis Shows Substantial Equivalence of the Protein Composition of Transgenic and Non-Transgenic Seed

Because Gly m Bd 30 K is a member of the papain superfamily of Cys proteases, we reasoned that it might possess some cryptic proteolytic or other un-
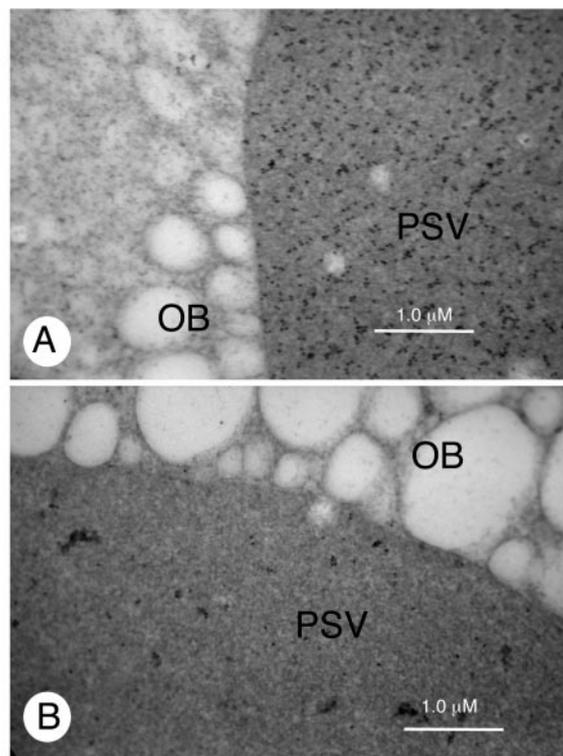

Figure 2. Immunogold assay of Gly m Bd 30 K cross-reactive proteins in silenced (A) and control (B) lines. A, Abundant Gly m Bd 30 K label in a late maturation soybean cell of a control. B, Similar transgenic soybean cell in which Gly m Bd 30 K accumulation was suppressed. OB, Oil bodies; PSV, protein storage vacuoles.

known activity involved in protein processing or turnover. To address the question of possible protein changes, a comparative two-dimensional gel/MS proteomic analysis capable of specifically quantifying and identifying individual polypeptides was performed on total mature seed protein extracts from Gly m Bd 30 K-silenced R3 seed and R3 control seed derived from R1 null segregants. Representative gel images (Fig. 3) from such an analysis illustrated a very similar pattern of protein features between the two samples. The features (spots) were visualized using a fluorescence dye (Page et al., 1999), and the images were digitally captured. This permitted quantification of polypeptides as percentage values of the total protein quantity of a sample. The separation of each sample was repeated three times, which enabled the application of statistical methods for the evaluation of quantitative data. This analysis was performed using the ROSETTA software package (OGS, Oxford) and resulted in the detection of 1,432 reproducible unique features at a level of more than 1 ng of protein. On the basis of the statistical analysis ($P = 0.05$), only five of these polypeptide features were found consistently different between the transgenic and control samples. Furthermore, all of these five polypeptides were absent in the Gly m Bd 30 K-silenced seed and were found in a close pI range
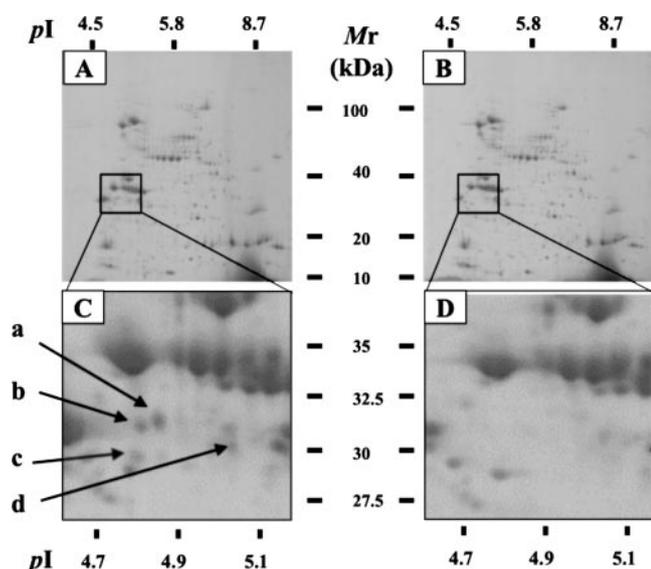
Figure 3. Two-dimensional protein analysis of control (A and C) and Gly m Bd 30 K-silenced (B and D) lines. Samples from mature seeds were fractionated on large-scale two-dimensional gels. The resulting gels were scanned, and differences between the gels were identified by automated analysis. The boxed areas on A (control line) and B (Gly m Bd 30 K-silenced line) are shown magnified in C and D, respectively. The Gly m Bd 30 K-silenced line (B and D) lacks several spots (C, a–d) present in the wild-type line (A and C) that are the consequence of the loss of isoforms and differential processing forms of Gly m Bd 30 K.

(4.5–5) and a relative mass between 25 and 35 kD, consistent with Gly m Bd 30 K-related polypeptides. In the individual samples, the changed polypeptides accounted for only about 0.1% to 0.2% of the total protein quantity detected in control seed. Tandem MS identification of the five differentially accumulated polypeptides and the identification of 145 abundantly expressed but non-differentially accumulated proteins was attempted and succeeded with the annotation of a total of 111 polypeptides (Fig. 4; Supplemental Table I, which can be viewed at www.plantphysiol.org). This amounted to an identification rate of 74% overall, and failures reflected primarily the incomplete coverage of the soybean proteins in sequence databases. The identified proteins in this study accounted together for 62% of the total protein quantity of each sample (Supplemental Fig. 1) and represented in its majority well-characterized soybean seed proteins (Nielsen and Nam, 1999). Two of the polypeptides (Fig. 3C, a and b) diminished in transgenic seed were identified by tandem MS as Gly m Bd 30 K-related polypeptides, corroborating the removal of this protein. Two other differentially accumulated polypeptides (Fig. 3C, c and d) were identified as human keratin and likely represent a contamination from sample handling. The identification of one differentially accumulated polypeptide was attempted twice but failed due to its low abundance and insufficient spectral coverage. Overall, the proteome analysis strongly supported a substantially

equivalent protein composition of wild-type and transgenic soybean seed.

DISCUSSION

Food allergies have been recognized as a growing problem. Increased diversity of diets and food sources have allowed much wider choices of food and with that much greater potential to encounter a food that elicits an immunological response. Among the major foods wheat, dairy, eggs, and soybean are often cited as major sources of food allergies due to their inclusion as significant fractions of all foods, especially prepared foods so often used in industrialized countries. Gly m Bd 30 K was selected as a model to eliminate a food allergy through the use of biotechnology because it is an immunodominant allergen in soybean, one of the most significant agricultural commodities. Soybean allergy has significant impact on sensitive people (Herian et al., 1990) particularly babies and toddlers. Soybean-based formula, the most frequently used non-dairy formula, is normally recommended when a baby exhibits milk sensitivity. The widespread use of these soybean-based formulas exposes a large fraction of babies to soybeans and potential soybean sensitivity (Heppell et al., 1987; Cantani and Lucenti, 1997). Gly m Bd 30 K has been shown to account for a majority (>65%) of IgE binding to soybeans tested with serum from several soy-sensitive humans (Yaklich et al., 1999). It has approximately 14 human IgE epitopes, some of which overlap (Helm et al., 1998). Soybean allergies are also found in many domesticated animals that are fed a large soybean component in their diet. Pigs, calves, and salmon are among the farm animals with significant soybean sensitivity (Barratt et al., 1978; Gardner et al., 1990; Li et al., 1990, 1991; Bailey et al., 1993; Friesen et al., 1993; Dreau et al., 1995a, 1995b; Nordrum et al., 2000). Other members of the papain protease superfamily have been implicated in allergic reactions. The fecal dust mite allergen Der 1p, for example, is a Cys protease (Simpson et al., 1989; Yasuhara et al., 2001). Among plant Cys proteases, the archtype papain evokes allergic responses in sensitive people from its use as meat tenderizer (Soto-Mera et al., 2000), in latex products (Quarre et al., 1995), in contact lens cleaner (Fisher, 1985), or in the juice used to make throat lozenges (Iliev and Elsner, 1997). The destructive proteolytic activity of the Cys proteases may be a significant factor in the allergenicity of these proteins (Chambers et al., 1998; Deleuran et al., 1998). However, the lack of the catalytic Cys in Gly m Bd 30 K (Kalinski et al., 1990, 1992) suggests that it is primarily structural determinants and not enzymatic activity that induces allergenicity in this case.

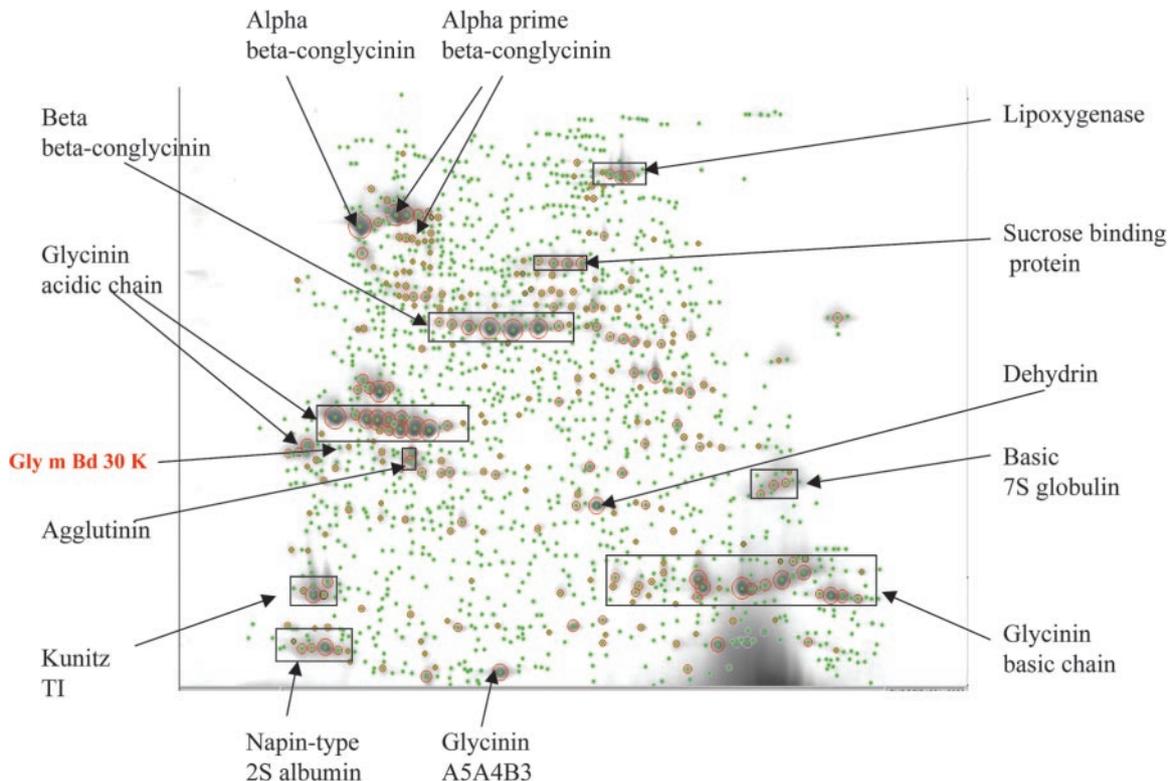Gly m Bd 30 K is almost uniformly distributed in

Figure 4. Two-dimensional overview map of proteins extracted from mature soybean seed. Composite image based on three independently conducted two-dimensional gel separations of seed protein extracts. Green circles indicate software-detected individual features (cut-off level, 1 ng of protein), and red circles indicate features that were selected and cut from gels for MS identification. On hundred and eleven1 features were identified (see Supplemental Table I). Major seed proteins and their isoforms are labeled (arrows).

the genetic diversity of soybean, and naturally occurring varieties lacking this protein have not been found (Yaklich et al., 1999). In addition, germplasm selection and mutation-breeding programs have failed to yield Gly m Bd 30 K-free soy protein (Samoto et al., 1997). We therefore used a biotechnology approach to remove this allergen in transgenic soybean seeds. To achieve this, we induced gene silencing of the endogenous Gly m Bd 30 K gene by reintroducing into the plant a copy of part of the Gly m Bd 30 K cDNA. The resulting gene silencing in the transgenic soybean seeds produced an apparent complete suppression of Gly m Bd 30 K protein. Homozygous Gly m Bd 30 K-suppressed plants have been selected and are presently being evaluated in field plots. The suppression of Gly m Bd 30 K does not appear to introduce any overt phenotype in the soybean plants, which complete their life cycle with no apparent differences in growth, development, reproduction, seed set, and seed maturation compared with the wild type.

Although a member of the papain superfamily of proteases and despite attempts to document a function (E.M. Herman, unpublished data), Gly m Bd 30 K has never been shown to possess any enzymatic activity. If it did exert any activity on proteins other than itself, it would be expected that this would show as an alteration of relative mass/pI of those proteins. The comparison of protein profiles from Gly m Bd 30 K suppressed and control seed using a sensitive two-dimensional electrophoresis method appeared to offer the best chance to uncover any cryptic Gly m Bd 30 K-specific proteolytic protein-processing events. Suppression of processing would have resulted in the accumulation of precursor molecules as new protein spots as well as in the disappearance of spots of mature processed polypeptides. However, this was not observed. The only soybean seed-specific protein difference observed during the two-dimensional/MS analysis was the diminution of Gly m Bd 30 K in the transgenic line. The lack of collateral shifts or processing of proteins in the Gly m Bd 30 K-silenced soybean seeds supports a lack of intrinsic proteolytic activity of the protein, probably caused by the substitution of a Gly for a conserved Cys in the catalytic site. Therefore, silencing Gly m Bd 30 K results in the elimination of a major allergen without inducing any detectable new proteins as a collateral consequence. One concern often expressed of plant biotechnology is the potential for introducing new allergens. A comparison of the Gly m Bd 30 K with other soybean lines using immunological assays with sera from soy-sensitive people shows that no new proteins with

IgE-immunological cross-reactivity were induced by silencing Gly m Bd 30 K.

The combination of the Gly m Bd 30 K-suppressed line with mutant lines lacking the other major soy allergens (Samoto et al., 1997; Ogawa et al., 2000) should result in a hypoallergenic soybean. For the majority (65%) of soy-sensitive individuals, who react only to Gly m Bd 30 K, the transgenic line alone will be hypoallergenic. The requirements for confirmation of presumed hypoallergenic status of foods with suppressed allergens include a number of consecutive steps. These include preparation of the hypoallergenic food, in vitro verification of claimed hypoallergenicity by SDS-PAGE immunoblotting, in vivo testing of the hypoallergenic food in sensitized animals, a skin prick test with the extract of the hypoallergenic food in sensitized subjects, and finally in vivo verification by administering the hypoallergenic food openly to established sensitized subjects. Using Gly m Bd 30 K-specific IgG antibodies from rabbits and antisera containing IgEs from soy-sensitive people, we have fulfilled the first two of these five criteria for Gly m Bd 30 K sensitivity. Experiments on soy-sensitive animal populations are currently under way, and in addition to providing support for the third step, they will provide an experimental basis for further human tests.

The genetic modification of plants by transgenic methods has raised the possibility of adding novel proteins that could potentially be human allergens (Yadav et al., 1993). In a recent study, Bhalla and coworkers (2001) demonstrated the converse by using genetic engineering to remove a pollen allergen, Lol p 5, from ryegrass. In our study, we have shown it is also possible to remove a major food allergen by gene-silencing techniques. Thus biotechnology now offers the prospect of eliminating many allergens that pose difficulties for sensitive people. Further research will explore the agronomic characteristics of the Gly m Bd 30 K-silenced soybean plants and examine the effects of Gly m Bd 30 K-suppressed soybean protein on soy-sensitive individuals.

MATERIALS AND METHODS

Construction of Gly m Bd 30 K-Silencing Vector

The entire coding region from a Gly m Bd 30 K cDNA (GenBank accession no. J05560) was amplified from a soy seed cDNA library (Yadav et al., 1993) in a standard PCR reaction on a GeneAmp PCR System (Applied Biosystems, Foster City, CA) using Pfu polymerase (Stratagene, La Jolla, CA) with the following primers: primer 1, 5′-GAATT_CGCGGCCGC-ATGGGTTTCCTTGTGT-3′, and primer 2, 5′-GAATTCGCGGCCGCTCAA-AGAGGAGAGTGA-3′.

The 3′ ends of these primers correspond to nucleotides 3 to 18 (primer 1) and 1,129 to 1,144 (primer 2) of the cDNA sequence described in GenBank accession no. J05560. This represents the complete Gly m Bd 30 K ORF from the start codon (3–5) to the stop codon (1,142–1,144). The resulting amplified Gly m Bd 30 K fragment was bound by the *Not*I sites included in the primer sequences (underlined above). The amplified fragment was digested with *Not*I and ligated to *Not*I-digested and phosphatase-treated plasmid pKS67 (Cahoon et al., 2000). This vector contained a unique *Not*I site for cloning of transgenes that was flanked by the promoter of the gene for the α-subunit of β-conglycinin, for seed-specific expression of transgenes (Beachy et al., 1985), and a β-phaseolin termination sequence (Beachy et al., 1985; Doyle et al., 1986). Bacterial selection was conferred by a hygromycin B phosphotransferase gene under control of the T7 RNA polymerase promoter, and plant selection was conferred by a second hygromycin B phosphotransferase gene under control of the cauliflower mosaic virus 35S promoter. The pKS67 vector containing the ORF of the Gly m Bd 30 K cDNA was designated pKS73 (Fig. 5).

Transformation and Screening of Somatic Soybean (*Glycine max*) Embryos

The ability to change the Gly m Bd 30 K content of soybean embryos by gene suppression was tested by preparing transgenic soybean somatic embryos and assaying for the presence of Gly m Bd 30 K protein. The vector pKS73 was transformed into soybean somatic embryos of soybean cv Jack using the particle bombardment method of transformation as described (Gritz and Davies, 1983; Cahoon et al., 2000, 2001). Three lines of transformed embryogenic clusters (3/1, 6/1, and 7/1) were removed from liquid culture and were placed on a solid agar media (SB103) containing no hormones or antibiotics. Embryos were cultured for 4 weeks at 26°C with mixed fluorescent and incandescent lights on a 16-h/8-h day/night schedule.

During this period, individual embryos were removed from the clusters and screened for their lack of Gly m Bd 30 K protein by protein-blot analysis (Cahoon et al., 2000). Embryos were frozen in liquid nitrogen, ground in a mortar, and extracted with sample buffer (0.125 M Tris-HCl, pH 6.8, containing 0.4% [w/v] SDS, 20% [w/v] glycerol, 4% [w/v] SDS, and 0.2% [w/v] 2-mercaptoethanol) at a ratio of 1:5 (w/v). Solubilized proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were probed with a rabbit Gly m Bd 30 K polyclonal antibody for initial screening, and putative null embryo lines were confirmed using a mouse Gly m Bd 30 K monoclonal antibody as described (Herman et al., 1990). Embryo lines containing reduced amounts of the
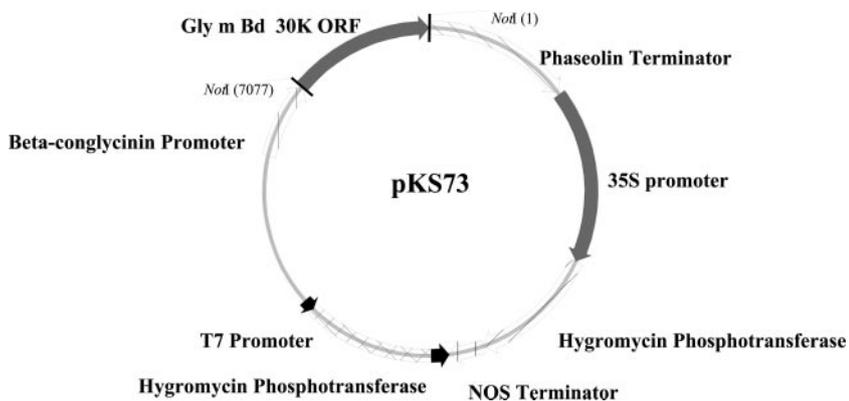

Figure 5. Map of Gly m Bd 30 K-silencing construct. Features indicated are the Gly m Bd 30 K-coding region, the β-conglycinin promoter, and the bean phaseolin termination region. Also shown are the transcriptional units for bacterial (T7) and plant (35S) hygromycin selectable marker genes.

endogenous Gly m Bd 30 K were regenerated into plants as previously described (Cahoon et al., 2001). Small chips taken from seeds of the resulting transgenic plants were screened for Gly m Bd 30 K protein as described above. Seed completely lacking Gly m Bd 30 K were planted to produce second and third generation plants that were homozygous for the Gly m Bd 30 K-suppressed phenotype.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining permission will be the responsibility of the requestor. Federal or International regulations may restrict shipment of viable transgenic seeds.

Electron Microscopic Immunocytochemistry

Immunolocalization of Gly m Bd 30 K in cotyledons of control and knockout plants was accomplished by a small modification of previously described procedures (Kalinski et al., 1992). Free-hand razor-blade cut sections of chips derived by cutting through the cotyledons of knockout and control seeds were obtained for non-destructive immunochemical and immunocytochemical analysis. The chips were hydrated for 14 h in an excess of water; each chip was then cut in half with one-half prepared for SDS-PAGE-immunoblot analysis as described above, and the other half was fixed in 4% (w/v) formaldehyde and 2% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The fixed chips were dehydrated in a graded ethanol series and embedded in LR White resin. Thin sections mounted on grids were labeled with anti-Gly m Bd 30 K monoclonal antibodies (Finer and McMullen, 1991) as ascites fluid diluted 1:100 in 5% (v/v) fetal bovine serum diluted in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% (w/v) Tween 20 (TBST) for 30 min at room temperature. The sections were then washed in TBST and indirectly labeled with 10 μg mL^{-1} rabbit anti-human IgE in TBST with 5% (v/v) fetal for 30 min at room temperature. The grids were then washed again in TBST, and then labeled with 10-nm colloidal gold particles coupled to anti-rabbit IgG (Ted Pella Inc, Tustin, CA) for 5 min. The grids were then washed in TBST and distilled water and then stained in 5% (w/v) uranyl acetate for 20 min. The grids were visualized in an electron microscope (400T, Philips, Eindhoven, The Netherlands) and photographed with an axial mounted CCD camera (Photometrics, Tuscon, AZ).

Two-Dimensional Gel Electrophoresis

Pooled mature seed from wild type (soybean cv Jack) and from transgenic Gly m Bd 30 K-suppressed plants, respectively, were frozen in liquid nitrogen, ground into a fine powder, defatted twice with hexane, and vacuum-dried. Protein was extracted by vortexing 100 mg of seed powder with 1,500 μL of extraction buffer (4% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 M urea, 2 M thiourea, 65 mM dithiothreitol, and 0.8% [w/v] Resolytes 3–10 [Bio-Rad, Hercules, CA]) for 5 min at room temperature and by clearing the extract by centrifugation at 13,000g for 5 min. The protein extracts were adjusted to a protein concentration of 1 mg mL^{-1}.

Two-dimensional electrophoreses was conducted at Oxford Glyco-Sciences (Oxfordshire, UK) as follows. Immobilized pH gradient gels (Immobiline 3–10NL, Amersham Biosciences, Uppsala) were rehydrated and focused overnight with 120 μg of solubilized protein according to Sanchez et al. (1997). The gels were fixed and stained with a fluorescent dye, and images were obtained exactly as described (Page et al., 1999). Three replica gels were run for each sample.

Primary images were processed, and individually resolved protein features were enumerated, quantified on the basis of fluorescence signal intensity, and compiled into a database table and into composite images by using proprietary software of Oxford GlycoSciences. A total of 1,432 unique protein features were identified in the wild-type and transgenic seed samples. The data obtained from the replica gels were statistically analyzed (variance) to remove out-lying features. In addition, the composite images of the gels were examined for artifacts to eliminate impacted features from

the analysis. The analysis to identify differentially expressed proteins was then undertaken using Rosetta proprietary software package (OGS).

Protein features of interest were analyzed at OGS by tandem MS as described (Page et al., 1999). This procedure first involved determining a precise mass of a protein by matrix-assisted laser-desorption ionization time of flight-MS. This was matched with all of the proteins of exactly the same mass in the SwissProt and Pioneer-DuPont databases. The protein was then digested with trypsin, and several individual peptide fragments from each protein were subjected to a second Quadrupole Time of Flight-MS. The mass of each tryptic fragment corresponded to a unique amino acid composition. The combination of precise mass and amino acid composition of a number of peptide fragments is unique for every protein known, if the peptide fragments are in the database the identification of the protein is close to definitive. As described by Page et al. (1999), the database scores were filtered according to their cross correlation score (Xcorr), normalized difference correlation score (.Cn), and compatibility with trypsin digestion. As is standard for the technique, peptides were only used for protein identification where the probability scores were very high (Xcorr $\geq$ 1.2 and .Cn $\geq$ 0.2).

LITERATURE CITED

Bailey M, Miller BG, Telemo E, Stokes CR, Bourne FJ (1993) Immunological unresponsiveness following active primary responses to proteins in the weaning diet of piglets. Int Arch Allergy Immunol **101:** 266–271

Barratt ME, Strachan PJ, Porter P (1978) Antibody mechanisms implicated in digestive disturbances following ingestion of soya protein in calves and piglets. Clin Exp Immunol **31:** 305–312

Beachy RN, Chen ZL, Horsch RB, Rogers SG, Hoffmann NJ, Fraley RT (1985) Accumulation and assembly of soybean beta-conglycinin in seeds of transformed petunia plants. EMBO J **4:** 3047–3053

Bhalla PL, Swoboda I, Singh MB (2001) Reduction in allergenicity of grass pollen by genetic engineering. Int Arch Allergy Immunol **124:** 51–54

Burks AW Jr, Brooks JR, Sampson HA (1988) Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. J Allergy Clin Immunol **81:** 1135–1142

Bush RK, Hefle SL (1996) Food Allergens. Crit Rev Food Sci Nutr **36:** 119–163

Cahoon EB, Marillia EF, Stecca KL, Hall SE, Taylor DC, Kinney AJ (2000) Production of fatty acid components of meadowfoam oil in somatic soybean embryos. Plant Physiol **124:** 243–251

Cahoon EB, Ripp KG, Hall SE, Kinney AJ (2001) Formation of conjugated 8,10-double bonds by 12-oleic-acid desaturase-related enzymes: biosynthetic origin of calendic acid. J Biol Chem **276:** 2637–2643

Cantani A, Lucenti P (1997) Natural history of soy allergy and/or intolerance in children, and clinical use of soy-protein formulas. Pediatr Allergy Immunol **8:** 59–74

Chambers L, Brown A, Pritchard DI, Sreedharan S, Brocklehurst K, Kalsheker NA (1998) Enzymatically active papain preferentially induces an allergic response in mice. Biophys Res Commun **1253:** 837–840

Deleuran M, Ellingsen AR, Paludan K, Schou C, Thestrup-Pedersen K (1998) Der p1 and p2 patch tests in patients with atopic dermatitis: evidence for both allergenicity and proteolytic irritancy. Acta Derm Venereol **78:** 241–243

Doyle JJ, Schuler MA, Godette WD, Zenger V, Beachy RN, Slightom JL (1986) The glycosylated seed storage proteins of *Glycine max* and *Phaseolus vulgaris*: structural homologies of genes and proteins. J Biol Chem **261:** 9228–9238

Dreau D, Lalles JP, LeJan C, Toullec R, Salmon H (1995a) Hypersensitivity to soybean proteins in early weaned piglets: humoral and cellular components. Adv Exp Med Biol **37:** 865–869

Dreau D, Lalles JP, Toullec R, Salmon HB (1995b) Lymphocytes are enhanced in the gut of piglets fed heat-treated soyabean proteins. Vet Immunol Immunopathol **47:** 69–79

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In Vitro Cell Dev Biol 27: 175–182

Fisher AA (1985) Allergic reactions to contact lens solutions. Cutis 36: 209–211

Friesen KG, Goodband RD, Nelssen JL, Blecha F, Reddy DN, Reddy PG, Kats LJ (1993) The effect of pre- and postweaning exposure to soybean meal on growth performance and on the immune response in the early-weaned pig. J Anim Sci 71: 2089–2098

Gardner RW, Shupe MG, Brimhall W, Weber DJ (1990) Causes of adverse responses to soybean milk replacers in young calves. J Dairy Sci 73: 1312–1317

Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. Gene 25: 179–188

Helm RM, Cockrell G, Herman E, Burks AW, Sampson HA, Bannon GA (1998) Cellular and molecular characterization of a major soybean allergen. Int Arch Allergy Immunol 117: 29–37

Helm RM, Cockrell G, West CM, Herman EM, Sampson HA, Bannon GA, Burks AW (2000) Mutational analysis of the IgE-binding epitopes of P34/Gly m1. J Allergy Clin Immunol 105: 378–384

Heppell LM, Sissons JW, Pedersen HE (1987) A comparison of the antigenicity of soya-bean-based infant formulas. Br J Nutr 58: 393–403

Herian AM, Taylor SL, Bush RK (1990) Identification of soybean allergens by immunoblotting with sera from soy-allergic adults. Int Arch Allergy Appl Immunol 92: 193–198

Herman EM, Melroy DL, Buckhout TJ (1990) Apparent processing of a soybean oil body membrane protein accompanies the onset of oil mobilization. Plant Physiol 94: 341–349

Iliev D, Elsner P (1997) Generalized drug reaction due to papaya juice in throat lozenges. Dermatology 194: 364–366

Kalinski AJ, Melroy DL, Dwivedi RS, Herman EM (1992) A soybean vacuolar protein (P34) related to thiol proteases which is synthesized as a glycoprotein precursor during seed maturation. J Biol Chem 267: 12068–12076

Kalinski AJ, Weisemann J, Matthews BF, Herman EM (1990) Molecular cloning of a protein associated with soybean oil bodies which is homologous to thiol proteases of the papain family. J Biol Chem 265: 13843–13848

Kinney AJ, Jung R, Herman EM (2001) Cosuppression of the α-subunits of β-conglycinin in transgenic soybean seeds induces the formation of endoplasmic reticulum-derived protein bodies. Plant Cell 13: 1165–1178

Li DF, Nelssen JL, Reddy PG, Blecha F, Hancock JD, Allee GL, Goodband RD, Klemm RD (1990) Transient hypersensitivity to soybean meal in the early-weaned pig. J Anim Sci 68: 1790–1799

Li DF, Nelssen JL, Reddy PG, Blecha F, Klemm R, Goodband RD (1991) Interrelationship between hypersensitivity to soybean proteins and growth performance in early-weaned pigs. J Anim Sci 10: 4062–4069

Nielsen NC, Nam Y-W (1999) Soybean globulins. In PR Shewry, R Casey, eds, Seed Proteins. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 685–708

Nordlee JA, Taylor S, Jones RT, Yuninger JY (1981) Allergenicity of various peanut products as determined by RAST inhibition. J Allergy Clin Immunol 68: 376–382

Nordrum S, Bakke-McKellep AM, Krogdahl A, Buddington RK (2000) Effects of soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss). Comp Biochem Physiol B Biochem Mol Biol 125: 317–335

Ogawa T, Bando N, Tsuji H, Nishikawa K, Kitamura K (1995) Alpha-subunit of beta-conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic dermatitis. Biosci Biotechnol Biochem 59: 831–833

Ogawa T, Bando N, Tsuji H, Okajima H, Nishikawa K, Sasaoko K (1991) Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic dermatitis. J Nutr Sci Vitaminol 37: 555–565

Ogawa T, Samoto M, Takahashi K (2000) Soybean allergens and hypoallergenic soybean products. J Nutr Sci Vitaminol 46: 271–279

Ogawa T, Tsuji H, Kitamura K, Zhu UL, Hirano H, Nishikawa K (1993) Identification of the soybean allergenic protein, Gly m Bd 30 K, with the soybean seed 34-kDa oil-body-associated protein. Biosci Biotechnol Biochem 57: 1030–1033

Page MJ, Amess B, Townsend RR, Parekh R, Herath A, Brusten L, Zvelebil MJ, Stein RC, Waterfield MD, Davies SC et al. (1999) Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammoplasties. Proc Natl Acad Sci USA 96: 12589–12594

Quarre JP, Lecomte J, Lauwers D, Gilbert P, Thiriaux J (1995) Allergy to latex and papain. J Allergy Clin Immunol 95: 922

Samoto M, Fukuda Y, Takahashi K, Tabuchi K, Hiemori M, Tsuji H, Ogawa T, Kawamura Y (1997) Substantially complete removal of three major allergenic soybean proteins (Gly m Bd 30 K, Gly m Bd 28K and the alpha-subunit of conglycinin) from soy protein by using a mutant soybean, Tohoku 124. Biosci Biotechnol Biochem 61: 2148–2150

Sanchez JC, Rouge V, Pisteur M, Ravier F, Tonella L, Moosmayer M, Wilkins MR, Hochstrasser DF (1997) Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients. Electrophoresis 18: 324–327

Simpson RJ, Nice EC, Moritz RL, Stewart GA (1989) Structural studies on the allergen Der p1 from the house dust mite Dermatophagoides pteronyssinus: similarity with cysteine proteinases. Protein Seq Data Anal 2: 17–21

Soto-Mera MT, Lopez-Rico MR, Filgueira JF, Villamil E, Cidras R (2000) Occupational allergy to papain. Allergy 55: 983–984

Taylor SL, Hefle SL (2001) Will genetically modified foods be allergenic? J Allergy Clin Immunol 107: 765–771

Thanh VH, Shibasaki K (1976) Major proteins of soybean seeds: a straightforward fractionation and their characterization. J Agric Food Chem 24: 1117–1121

Tsuji H, Okada N, Yamanishi R, Bando N, Kimoto M, Ogawa T (1995) Measurement of Gly m Bd 30 K, a major soybean allergen, in soybean products by a sandwich enzyme-linked immunosorbent assay. Biosci Biotechnol Biochem 59: 150–151

Vidal C, Perez-Carral C, Chomon B (1997) Unsuspected sources of soybean exposure. Ann Allergy Asthma Immunol 79: 350–352

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Perez-Grau L, Kinney AJ, Hitz WD, Booth JR, Schweiger B, Stecca KL et al. (1993) Cloning of higher plant omega-3 fatty acid desaturases. Plant Physiol 103: 467–476

Yaklich R, Helm R, Herman E (1999) Analysis of the distribution of the major soybean allergen in a core collection of Glycine max accessions. Crop Sci 39: 1444–1447

Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y (2001) Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding. Clin Exp Allergy 31: 116–124