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Soybean Allergenicity and Suppression of the Immunodominant Allergen

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ABSTRACT

The wide-spread use of soybean [*Glycine max* (L.) Merr.] products in processed foods poses a potential threat to soybean-sensitive, food-allergic individuals. Clinical symptoms of soybean allergy can be manifested as gastric distress or atopic dermatitis and, while usually not life threatening, suspected cases of anaphylaxis have been reported. In vitro assays of soybean seed proteins with sera collected from soybean-sensitive individuals have shown that major storage proteins as well as other minor seed proteins account for IgE binding. Gly m Bd 30k, a member of the papain superfamily of cysteine proteases, also referred to as P34, has been identified as a major allergen in soybean seeds. We have used gene silencing to eliminate accumulation of P34/Gly m Bd 30k in transgenic soybean. These transgenic plants, producing P34/Gly m Bd 30k-null seeds, lacked any obvious developmental or phenotypic differences when compared with control plants. The production of a P34/Gly m Bd 30k-null line eliminates one of the primary allergens present in soybean seeds.

Seeds Contain a Variety of Stored Proteins

Seeds contain a few abundant storage proteins encoded by a few large gene families as well as numerous other lower abundance proteins (see Herman and Larkins, 1999, for review). Many of these lower abundance seed proteins are involved either in hydrolysis of one of the many reserve substances or in defense against macro- or microorganisms utilizing those rich reserves of nutritive substances as sources of food. Various lower abundance ancillary proteins play significant roles in the seed's life cycle, but also in many cases, they either enhance or diminish utilization of seeds for human and animal consumption. Seeds contain factors that are either antimetabolic compounds or elicitors of food allergies. Soybean contains an array of antimetabolic compounds such as phytin, trypsin inhibitors, and oligosacchrides as well as proteins that elicit allergenic responses inducing adverse gastric responses and atopic dermatitis in sensitive humans and animals (Fig. 1).

P34 Is a Member of the Papain Superfamily Localized in Protein Storage Vacuoles

An abundant protein associated with the oil body fraction was purified from mature soybean seeds (Herman et al., 1990). Monoclonal antibodies against the oil body associated proteins included a 34-kDa protein termed P34. This P34 was highly enriched and partially purified from the oil body fraction. Initially, it was assumed to be a constituent of this organelle. Subsequent results demonstrated that P34 was localized in the seed protein storage vacuoles (Kalinski et al., 1992). P34 has a tendency to bind to lipid and oil that is likely significant in its subsequently demonstrated role as an allergen and as a possible defense protein (Okinaka et al., 2002).

A cDNA for P34 was isolated a λ− Zap phage library prepared soybean seed midmaturation mRNA using immunological screening with a monoclonal antibody (Kalinski et al., 1990). The deduced sequence exhibited high homology to members of the papain superfamily of cysteine proteases (Fig. 2). All cysteine proteases have highly conserved amino acids distributed throughout the complete sequence. Among these are disulfide bridges and glycine–glycine residues important for folding, and conserved amino acids in the active site that includes a cysteine and a histidine. P34 lacks the cysteine in the active site that is present in all other members of the papain superfamily. Another isolate of P34 cDNA (GenBank# AB013289) also lacks this conserved cysteine. This indicates that a key characteristic of this protein is the alteration of an otherwise completely conserved cysteine, which is the catalytic amino acid to a glycine. This suggests that P34 may not possess intrinsic proteolytic activity, and this is supported by protein engineering experiments whereby other members of the cysteine protease family have been synthesized in heterologous cells with site-directed mutagenesis changing the catalytic cysteine to serine and inactivating the protease (Rowan et al., 1992). We do not yet know if any other plant contains a homolog of P34 defined as a cysteine protease without a catalytic cysteine.

Like other cysteine proteases (North, 1986; Cigic et al., 2000; Tao et al., 1994; Wiederanders, 2000), P34 is initially synthesized as a large precursor with a 122 amino acid precursor domain that includes the signal sequence for translocation into the endoplasmic reticulum (ER) lumen. In vitro and in vivo synthesis and processing experiments have demonstrated that proP34 is produced as a glycoprotein with the utilized glycosylation site in the pro-domain (Kalinski et al., 1992). Cysteine proteases have both intracellular and extracellular roles with plant intracellular forms possessing an intrinsic targeting sequence NPIR (Holwerda et al., 1992) and closely-related sequences in plants such as the aligned NYIR in P34. The NPIR sequence is recognized by a *trans*-golgi receptor (BP80; AtELP) that targets proteins into the vacuole (Ahmed et al., 1997; Paris et al., 1997). Immunogold immunocytochemistry has localized P34 in the Golgi and Golgi-derived secretion vesicles transporting the protein to the protein storage vacuole. Extracellular forms of cysteine proteases, such as those secreted by cereal aleurone, probably default to the cell surface.

Cysteine proteases have many functional roles in both physiological and developmental events (Turk et al., 1996, 1997 for reviews). In particular, they are involved

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in cellular remodeling and programmed cell death (Ueda et al., 2000; Solomon et al., 1999; Griffiths et al., 1997), development (Koehler and Ho 1990; Mikkonen et al., 1996; Nong et al., 1995; Jones et al., 1996; Watanabe et al., 1991; Kalinski et al., 1997), stress response (Jones and Mullet, 1995; Khanna-Chopra et al., 1999; Koizumi et al., 1993), and plant defense (El Moussaoui et al., 2001; Linthorst et al., 1993). Among all proteins, P34 remains unique because of its active site modification and unknown function in soybean.

P34 May Have a Function in Pathogen Resistance

P34 may function as a pathogen resistance (PR) protein. Vacuolar PR proteins are abundant ancillary proteins in seeds, and they are inducible in vegetative tissues challenged with either pathogens or elicitors. A supporting evidence for this role is that the protein can be affinity-purified from a soybean leaf extract using an elicitor syringolipid on the bound ligand (Cheng et al., 1998). The presence of P34 in vegetative cells is correlated with the *RPG4* locus for pathogen resistance suggesting the potential role of P34 as a pathogen resistance protein. Immunoblot assays show that P34 protein is more abundant in *Pseudomonas*-resistant *RPG4* lines than in *Pseudomonas*-sensitive *rpg4* lines. Using two-hybrid analysis, a protein with specific binding to P34–syringolide complex has been isolated (Okinaka et al., 2002). How might this interaction be involved in a signaling response to infection requires further investigation. Demonstrating that P34 is actually involved in *Pseudomonas* resistance is yet to be done. The null of seed-specific expression of P34 now available (Herman et al., 2003) may prove useful to test the capacity of P34 to impede pathogen stress.

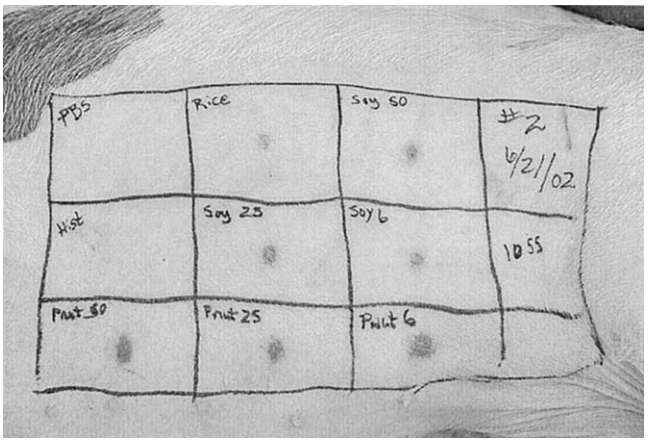

Fig. 1. Demonstration of the atopic skin response of a swine previously sensitized with peanut and soybean proteins. Photograph courtesy of Agricultural Research Magazine.

P34 Is the Immunodominant Allergen in Soybean Seed

Food allergies have been recognized as a growing problem. Increased diversity of diets and food sources have allowed wider choices of food, and with that, a greater

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1                                                          60
Actinidin     (1) -MGLPKSFVSMSLLFFSTLLILSLAFNAKNLTQRTN------DEVKAMYESWLIKYGKSY
  Der p 1     (1) -MKIVLAIASLLALS----------AVYAR----------PSSIKTFEEYKKAFNKSY
      P34     (1) MGFLVLLLFSLLGLSSSSSISTHRSILDLDLTKFTT-----QKQVSSLFQLWKSEHGRVY
   Papain     (1) -MAMIPSISKLLFVAICLFVYMGLSFGDFSIVGYSQNDLTSTERLIQLFESWMLKHNKIY
                  61                                                      120
Actinidin    (54) NSLGEWERRFEIFKETLRFIDEHNADTNR--SYKVGLNQFADLTDEEFRSTYLGFTSGSN
  Der p 1    (38) ATFEDEEAARKNFLESVKYVQSN--GG--------AINHLSDLSLDEFKNRFLMSAEAFE
      P34    (56) HNHEEEAKRLEIFKNNSNYIRDMNANRKSPHSHRLGLNKFADITPQEFSKKYLQAPKDVS
   Papain    (60) KNIDEKIYRFEIFKDNLKYIDETNKKNN---SYWLGLNVFADMSNDEFKEKYTGSIAGNY
                  121                                                     180
Actinidin   (112) KTKVSNRYEPRFG-----QVLPSYVDWRSAGAVVDIKSQGECGGCWAFSAIATVEGINKI
  Der p 1    (88) HLKTQFDLNAETNACSINGNAPAEIDLRQMRTVTPIRMQGGCGSCWAFSGVAATESAYLA
      P34   (116) QQIKMANKKMKKEQYSC-DHPPASWDWRKKGVITQVKYQGGCGRGWAFSATGAIEAAHAI
   Papain   (117) TTTELSYEEVLNDGD---VNIPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKI
                  181                                                     240
Actinidin   (167) VTGVLISLSEQELIDCGRTQNTRGCNGGYITDGFQFIINNGGINTEENYPYTAQDGECNL
  Der p 1    (148) YRNQSLDLAEQELVDCASQH---GCHGDTIPRGIEYIQHNG-VVQESYYRYVAREQSCRR
      P34    (175) ATGDLVSLSEQELVDCVEES--EGSYNGWQYQSFEWVLEHGGIATDDDYPYRAKEGRCKA
   Papain    (174) RTGNLNEYSEQELLDCDRRS--YGCNGGYPWSALQLVAQYG-IHYRNTYPYEGVQRYCRS
                  241                                                     300
Actinidin   (227) DLQNE------KYVTIDTYENVPYNNEWALQTAVTYQPVSVALDAAGDAFKHYSSGIFTG
  Der p 1    (204) PNAQR------FGISNYCQIYPPNVNKIREALAQTHSAIAVIIGIKDLDAFRHYDGRTII
      P34    (233) NKIQDKVTIDGYETLIMSDESTESETEQAFLSAILEQPISVSIDAK--DFHLYTGGIYDG
   Papain    (231) REKGP------YAAKTDGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGIFVG
                  301                                                     360
Actinidin   (281) P---CGTAIDHAVTIVGYGTEGGIDYWIVKNSWDTTWGEEGYMRILRNVG-GAGTCGIAT
  Der p 1    (258) QRDNGYQPNYHAVNIVGYSNAQGVDYWIVRNSWDTNWGDNGYGYFAANID-----LMMIE
      P34    (291) ENCTSPYGINHFVLLVGYGSADGVDYWIAKNSWGEDWGEDGYIWIQRNTGNLLGVCGMNY
   Papain    (285) P---CGNKVDHAVAAVGYGPN----YILIKNSWGTGWGENGYIRIKRGTGNSYGVCGLYT
                  361                                                 405
Actinidin   (337) MPSYPVKYNNQNHPKPYSSLINPPAFSMSKDGPVGVDDGQRYSA-
  Der p 1    (313) EYPYVVIL-------------------------------------
      P34    (351) FASYPTKEE--------SE----TLVSARVKGHRRVDHSPL----
   Papain    (338) SSFYPVKN-------------------------------------
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Fig. 2. The sequence alignment of four allergenic members of the papain family of cysteine proteases is shown.

potential for an individual to encounter a food that elicits an adverse immunological response. Among the major foods, wheat, dairy, eggs, and soybean are often cited as major sources of food allergies because of their inclusion as significant fractions of all foods especially prepared foods so often used in industrialized countries. Although allergies against major food sources do not usually provoke highly-dangerous anaphylaxis reactions as with peanut, tree nuts, and shellfish consumption, they are difficult to avoid as they are widely used in a variety of processed foods and food products, and cross-contamination with unlabeled allergens has been recognized as a growing problem (Vidal et al., 1997; Herian et al., 1993; Nordlee et al., 1981).

Soybean allergies are manifested as the consequence of inhalation and ingestion with the primary symptoms including asthma, atopic response (hives), and gastric distress. Inhalation allergy is largely occupational from dust, although this allergy has also been shown to be manifested from exposure to bean-bag chairs (Falleroni and Zeiss, 1996). Allergies arising from consumption can occur not only from a processed food like pizza (Senna et al., 1998), but also from other exposures such as cosmetics (Shaffrali and Gawkrodger, 2001). Widespread use of soybean-based formula exposes infants to soybean, and potential sensitivity to soybean (Heppell et al., 1987). Soybean-based formula is often used when a baby exhibits milk sensitivity. Soybean allergies are also common among farm animals with neonatal pigs among those most affected (Bailey et al., 1993; Barratt et al., 1978; Dreau et al., 1995a, 1995b; Friesen et al., 1993; Li et al., 1990; 1991). Other soybean sensitive animals include salmon and trout grown in aquaculture (Buttle et al., 2001; Nordrum et al., 2000), and young cattle (Gardner et al., 1990).

P34 was recognized as a major soybean allergen by Ogawa et al. (1991, 1993) after surveying proteins that bound IgEs derived from soybean-sensitive individuals. They determined the amino terminal sequence of an about 30-kDa IgE binding protein, and found that it corresponded exactly with P34, designated as Gly m Bd 30k. Human allergenicity to P34/Gly m Bd 30k is demonstrated by SDS/PAGE and immunoblots confirming it as an immunodominant human allergen (Fig. 3).

As a cysteine protease human allergen, P34/Gly m Bd 30k is not unique as the fecal dust mite allergen Der1p, also a cysteine protease, has been extensively characterized (Topham et al., 1994; Yasuhara et al., 2001). The primary cysteine protease of kiwi fruit, actinidin, is a food allergen for sensitive people (Pastorello et al., 1998). Other cysteine proteases induce occupational and food allergies when proteases of plant origin are used in the food industry (e.g., Nettis et al., 2001; Soto-Mera et al., 2000). An allergy to papain (Chambers et al., 1998; Niinimaki et al., 1993; Quarre et al., 1995), the archetype cysteine protease, occurs in individuals when their contact lenses are dipped in a solution to strip them from proteins (Bernstein et al., 1984; Fisher 1985; Santucci et al., 1985). Similar allergenic reactions to papain are observed on exposure to fruit juices in throat lozenges (Iliev and Elsner, 1997) and to meat tenderizer (Mansfield and Bowers 1983). One model for allergenicity of

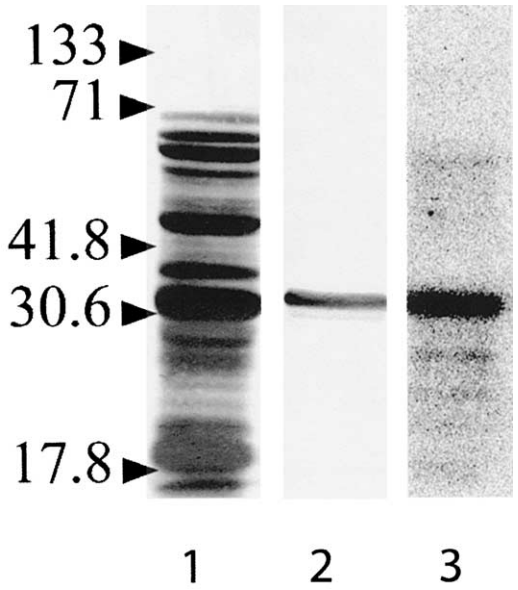

Fig. 3. A SDS/PAGE immunoblot of total soybean proteins (top panel) probed with a monoclonal antibody against P34/Gly m Bd 30k (middle panel) and sera from a human baby with soybean sensitivity (bottom panel) is shown. These blots show that P34/Gly m Bd 30k is the primary or immunodominant allergen.

the papain superfamily is that the destructive proteolytic activity has a major role in inducing the immune response to these proteins (Chambers et al., 1998; Deleuran et al., 1998; Yasuhara et al., 2001) that would not apply to P34/Gly m Bd 30k because of lack of an intrinsic protease activity.

The human immunology of P34/Gly m Bd 30k in soybean-sensitive individuals has been investigated in great detail by Helm et al. (1998, 2000). Human IgE epitopes of P34/Gly m Bd 30k from soy-sensitive people have been determined by solid-phase immunoassay using offset synthetic peptides corresponding to the entire open reading frame. These assays indicate that there are 14 epitopes distributed across the mature P34/Gly m Bd 30k sequence. The individual epitopes vary in intensity with some epitopes deemed "hotter" than others. The same procedure has been used to assess immunological differences among unrelated soy-sensitive individuals. It has been shown that although P34/Gly m Bd 30k is an immunodominant allergen, individual IgE epitopes exhibit varying intensities among individuals, although some epitopes are predominantly stronger in most tested individuals (Helm et al., 1998). Immunodominant epitopes have been analyzed further by inducing glycine mutations within the epitope to map critical amino acids (Helm et al., 2000). Among six epitopes analyzed, the antigenicity is alleviated by glycine substitution in any position in one epitope, while in another epitope, the immuno-reactivity is not altered, and only a single critical amino acid is observed in the remaining four epitopes.

Production and Isolation of Transgenic Soybean Lines with Suppressed P34/Gly m Bd 30 k Accumulation

For soybean with minor proteins accounting for most of the allergenicity observed, screening naturally oc-

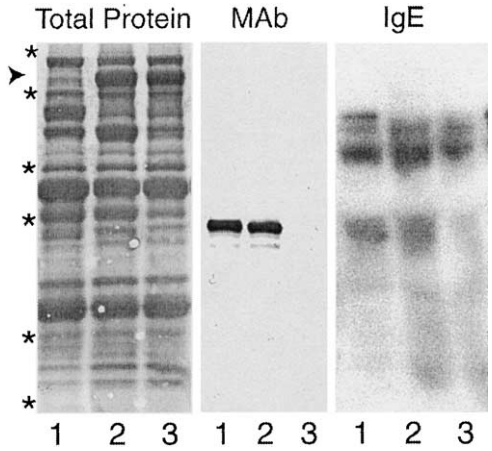

Fig. 4. The suppression of P34 by sense cosuppression is shown in the adjacent blots. The three panels show replicate samples stained for total protein with amido black, and labeled with either anti-P34 monoclonal antibody or with IgEs from a soybean-sensitive person. Lane 1 is a transgenic control of another soybean suppressing the α/α' conglycinin (arrowhead) that does contain P34 (Kinney et al., 2001). Lane 2 is a transgenic soybean line containing a construct that includes the P34 cDNA that did not induce suppression. Lane 3 is a T3 generation homozygous transgenic soybean with the P34 construct inducing the complete suppression of P34 accumulation. Note the complete absence of P34 assayed by the monoclonal antibody and IgE cross-reactivity using sera from a soybean-sensitive person. Reprinted from Plant Physiol. 132: 36-43 (2003).

curring variants present in seed collections might allow for identification of lines with either reduced or modified allergenicity. Yaklich et al. (1999) surveyed a small collection of diverse soybean cultivars and wild soybean relatives with immunoblots and a sera pool from soybean-sensitive individuals. The major soybean allergen Gly m Bd 30k was abundant in all domesticated soybean; while, minor allergenic proteins showed some variability, and a conglycinin null was identified. Among other minor soybean allergens, such as trypsin inhibitors, a naturally occurring null was identified. Among wild relatives of soybean, a wider variability in allergenicity was observed. The use of sera from soybean-sensitive individuals to probe protein blots of wild relatives of domesticated soybean revealed proteins intensely bound to IgEs. Allergen-null lines of allergenic crops would be desirable and could be used to produce hypoallergenic products; e.g., soybean-based infant formula, dog food, swine, calf, and fish feed.

The use of targeted allergen suppression has been successful in obtaining a soybean line lacking the immunodominant human allergen Gly m Bd 30k (Herman et al., 2003). Sense cosuppression was used to produce a knockout of seed allergen. P34/Gly m Bd 30k cDNA was cloned into a vector in the sense direction with the conglycinin promoter to control expression. Transformation was accomplished by particle bombardment of embryogenic suspension culture of soybean cv. Jack. After selection, embryos were regenerated and assayed for P34/Gly m Bd 30k content using a monoclonal antibody. Several lines with apparent gene suppression were identified, as well as other lines with apparent overaccumulation. Soybean plants were regenerated, and plants and lines with little or no apparent P34/Gly m Bd 30k content were identified. Additional generations and selections were assayed, and by the T4 generation, homozygous plants exhibiting stable seed-specific suppression of P34/Gly m Bd 30k accumulation were obtained.

Complete suppression of P34/Gly m Bd 30k is observed (Fig. 4). Soybean plants with suppressed seed-specific P34/Gly m Bd 30k have no apparent phenotypic changes; they germinate, grow, flower, set pod, and seed normally. The subcellular structure of the protein storage vacuoles where the P34/Gly m Bd 30k is not altered in the suppressor, except for the absence of P34/Gly m Bd 30k. The P34/Gly m Bd 30k suppressed seeds have been subjected to 2D gels and proteomic analysis to determine whether collateral changes in protein content may have occurred. Analysis of over 1400 different polypeptides in control (nontransgenic) seed and P34/Gly m Bd 30k-suppressed seeds have demonstrated the only apparent change in the polypeptide composition is that of the targeted antigen. This indicates that the P34/Gly m Bd 30k-suppressed soybean are substantially equivalent to control soybean (Herman et al., 2003).

To assure safety of genetically engineered (GE)-produced products, there has been increased emphasis on substantial equivalence to demonstrate that the GE modification does not produce other unintended collateral changes. Any genetic modification whether by directed procedures of recombinant biology, mutation, or by conventional breeding may alter the quantity of likely allergenic proteins. Recently, soybean lines suppressing either the 7S conglycinin or the 11S glycinin have been hybridized producing a hybrid line suppressing both major storage proteins. Analysis of seeds of this hybrid have revealed that the P34/Gly m Bd 30k allergen appears to be up-regulated, becoming one of the major proteins present in these seeds (Takahashi et al., 2003).

Soybean seeds with suppressed P34/Gly m Bd 30k offer an opportunity to mitigate soybean food allergies in humans and animals. Research is in progress to evaluate the impact of these soybeans in mitigating gastrointestinal problems in neonatal pigs, and produce a model system for studying food allergies using the tools of biotechnology.

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