J. Plant Biol. (2010) 53:113–120
DOI 10.1007/s12374-009-9094-y

ORIGINAL RESEARCH

Assessment of Phosphinothricin Acetyltransferase (PAT) Degradation From Transgenic Zoysiagrass Digested with Simulated Gastric Fluid (SGF)

Hyeon-Jin Sun · Hong-Gyu Kang · Tae-Woong Bae · Tae-Gun Cho · Joonki Kim · Pyung-Ok Lim · Key-Zung Riu · Hyo-Yeon Lee

Received: 4 November 2009 / Revised: 16 December 2009 / Accepted: 21 December 2009 / Published online: 4 February 2010

Abstract The in vitro pepsin digestion assay is the international standard for assessing the safety or risk of novel proteins newly produced in transgenic crops. However, this protocol, based on the degradation of protein purified from *Escherichia coli*, has recently been criticized for problems such as its objective detection limit. Here, we estimated the digestion stability of the phosphinothricin acetyltransferase (PAT) protein in soluble proteins as well as from leaf tissue powder in simulated gastric fluid (SGF). Our line of genetically modified zoysiagrass carried a single copy of the *bar* gene, which entered a chromosomal region not encoding protein. We designated it as Jeju Green 21 (JG21). From total soluble proteins extracted from JG21 leaves, digestibility of the PAT protein in SGF was examined by enzymatic assays, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western gel blots, and an immunodetection strip kit. The degradability of pure PAT protein obtained from *E. coli* was clearly apparent within at least 30 s. However, PAT degradation in leaf tissue powder was significantly delayed, indicating that some matrices in that powder might have influenced its digestion stability by SGF. Nevertheless, degradation of the powder (real-life) sample was complete within at least 5 min, suggesting that this protein produced in JG21 zoysiagrass can be digested harmlessly in the stomachs of humans or livestock.

Keywords GM crop · PAT · Safety assessment · Simulated gastric fluid (SGF) · Zoysiagrass

H.-J. Sun and H.-G. Kang contributed equally to this work.

H.-J. Sun · H.-G. Kang (✉) · T.-W. Bae · T.-G. Cho · J. Kim · P.-O. Lim · K.-Z. Riu · H.-Y. Lee
Subtropical Horticulture Research Institute,
Jeju National University,
Jeju 690-756, South Korea
e-mail: honggyu@cheju.ac.kr

K.-Z. Riu · H.-Y. Lee (✉)
Faculty of Biotechnology, Jeju National University,
Jeju 690-756, South Korea
e-mail: hyoyeon@cheju.ac.kr

P.-O. Lim
Department of Science Education, Jeju National University,
Jeju 690-756, South Korea

Introduction

The *bar* gene, originally cloned from *Streptomyces hygroscopicus*, encodes phosphinothricin acetyltransferase (PAT; Thompson et al. 1987). In plant transformations, *bar* is used as a selectable marker gene and as a reporter gene in chimeric gene constructs (Wehrmann et al. 1996). For commercial purposes, it has been introduced into corn (*Zea mays*; T25), cotton (*Gossypium hirsutum*; LLCotton25), rice (*Oryza sativa*; LLRICE06), soybean (*Glycine max*; W98), and canola (*Brassica* sp.; RF1) to confer the herbicide tolerance trait to transgenic plants (http://bch.cbd.int/database/organisms/).

Genetically modified (GM) crops can be commercialized after they are approved as environmentally friendly and safe to use as a food source. This approval is gained through various science-based investigations, e.g., environmental and human health risk assessments, that fulfill regulations drafted for living genetically modified organisms (Nap et al. 2003; Teng 2008). Safety evaluations related to food quality or human health follow recommendations provided by various authorities and international organizations, including FAO/WHO, *Codex alimentarius*, and OECD

(Herouet et al. 2005). Assessments of transgenic plants that carry foreign proteins such as PAT are largely based on evaluating both the protein itself and the transgenic plant. For this protein, tests are designed to monitor food toxins, allergens, degradation by gastric/intestinal fluids, etc. (Wehrmann et al. 1996; Herouet et al. 2005; Ofori-Anti et al. 2008). The PAT protein loses its activity at a pH below 4 or by heating for 30 min (Wehrmann et al. 1996; Herouet et al. 2005). In addition, it causes no significant health risks to livestock or human health (Jones and Maryanski 1991; Sjoblad et al. 1992; Schmidt 1994; Mossinger and Dietrich 1998; Thomas et al. 2004; Herouet et al. 2005). Its amino acid sequences show less than 35% homology with known allergenic sequences (Bae et al. 2008). Finally, no homology has been found between its sequences and those of eight amino acid allergen epitopes (Bae et al. 2008).

Turfgrass is broadly categorized as a crop because of its enormous economic value, although its cultivation is mainly for purposes other than food production, livestock fodder, or fuel (http://en.wikipedia.org/wiki/Crop). Zoysiagrass (*Zoysia japonica* Steud.) is a warm-season turfgrass indigenous to temperate regions such as Korea, Japan, and China. This perennial overwinters in a dormant state with dry brown leaves and stems. Its popularity has now expanded globally, including to the USA, and it is utilized in courtyards, parks, golf courses, and athletic fields because of its tolerances to drought and disease and its relatively slow growth habit (Bae et al. 2008). Transgenic herbicide-tolerant zoysiagrass carrying the *bar* gene has been produced via GM crop technology by Toyama et al. (2003). Because of ecological and environmental concerns, Bae et al. (2008) have performed a biosafety assessment that is based on allergic reaction tests with pollen extracts. There, no difference has been found between GM and wild-type plants.

The effect of GM crops on human health and/or the environment is difficult to determine with current scientific tools. Therefore, the governing of any potential risk depends on science-based regulations that are strictly applied, regardless of the particular plant species or how it is to be utilized in cultivation. Here, we conducted in vitro pepsin digestion assays for the degradation of PAT protein as part of the safety evaluation of GM herbicide-tolerant zoysiagrass. As well as evaluating the stability of PAT as produced in *Escherichia coli* (the standard test), we also monitored its performance when leaf tissue powder was treated with simulated gastric fluid (SGF).

Materials and Methods

DNA Gel Blot Analysis

The T-DNA region of the pGPTV-HB binary vector (Lee et al. 1998; Toyama et al. 2003) was completely sequenced by a biotechnology company (GENOTECH, Korea). For DNA blot analysis, genomic DNA was extracted from fresh young leaves of zoysiagrass (*Z. japonica* Steud.) according to the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1985). About 10 μg of DNA was digested with *Hin*dIII, *Xho*I, or *Eco*RI, separated on a 1% agarose gel, and transferred to a Hybond-N^+ nylon membrane (GE Healthcare). A digoxigenin-labeled *bar* gene-specific probe was generated with a polymerase chain reaction (PCR) DIG probe synthesis kit (Roche) and Bar-F and Bar-R primers (Table 1), according to the manufacturer's protocol. The blot was hybridized in a high sodium dodecyl sulfate (SDS) buffer for 20 h at 60°C (Church and Gilbert 1984; Kang et al. 1997). Afterward, it was washed

Table 1 PCR primers used in this study

Name	Sequence 5′ to 3′	Size (bp)
Bar-F	TTTCCATGGCTATGAGCCCAGAACGACGC	29
Bar-R	TTGGATCCTCAGATCTCGGTGACGGGCAG	29
RB1	TGGCTCCTTCAACGTTGCGGTTC	23
JGRB1	AAGATCGGTTTGACTGAGCTGAG	23
RB2	GGCGAATGAGCTTGCATGCC	20
JGRB2	ACGGCATCATCGTCGCATGCC	21
JGLB1	TGACCTAATCGCGCGCTGATCG	22
JGLB2	CATGACCTAAGTTCATCCGTACC	23
SRB1	CGCCAGCTGGCGTAATAGCG	20
SRB2	ACGGCTTGTCCCGCGTCATC	20
SLB1	ATTTATGGAACGTCAGTGGAGCA	23
SLB2	GAACGCGCAATAATGGTTTCTG	22
AD2	NGTCGA(G/C)(A/T)GANA(A/T)GAA	16
AD3	(A/T)GTGNAG(A/T)ANCANAGA	16

twice in 0.2× saline sodium citrate, 0.1% SDS for 15 min at 60°C. Signals were detected by chemiluminescence, using CDP^{star} (Roche). The membranes were then exposed to X-ray film.

Segregation Analysis of the T-DNA

Segregation analysis of the T-DNA locus in our T_1 generation was performed by PCR with JGRB1, JGLB1, JGRB2, and JGLB2 primers (Table 1; Fig. 2b, c) from sequences flanking the T-DNA insertion site, and with RB1 and RB2 primers (Table 1; Fig. 2b, c) from sequences around the right and left borders of T-DNA. We also confirmed herbicide tolerance by spraying plants with Basta (Bayer CropScience, Australia) at a final concentration of 0.1% (*w*/*v*) glufosinate.

Isolation of Sequences Flanking the T-DNA Insertion Locus

Each DNA flanking the right and the left borders of the T-DNA insertion site was isolated by thermal asymmetric interlaced (TAIL)-PCR (Liu et al. 1995). Genomic DNA was prepared per the CTAB method. Two arbitrary degenerate primers (AD2 and AD3; Liu et al. 1995) and four specific primers (SRB1, SRB2, SLB1, and SLB2) were synthesized (Table 1). The amplified DNA fragments were sequenced and analyzed by online programs serviced by NCBI (http://www.ncbi.nlm.nih.gov/).

Production and Purification of Protein from *E. coli*

Based on the T-DNA sequences of pGPTV-HB, a pair of specific primers was designed for amplifying *bar*—Bar-F and Bar-R (Table 1). PCR conditions included heating at 95°C for 5 min, then 35 cycles of amplification (95°C for 1 min, 60°C for 1 min, and 72°C for 1 min), followed by final extension at 72°C for 10 min. Genomic DNA from herbicide-tolerant zoysiagrass was used as template. The resulting PCR fragments were confirmed by sequencing and cloned into the *Nco*I/*Bam*HI sites of pET-30a (+) (Novagen), an expression vector, using the *E. coli* BL21 strain. The recombinant bacterium was grown in Luria broth (Sambrook et al. 1989) containing kanamycin (50 μg ml^{-1}) for selection. PAT protein was induced with isopropyl β-D-thiogalactopyranoside and purified by using a His·Bind® Kit (Novagen) according to the manufacturer's instructions.

Acetyltransferase Assay

PAT activity was measured by monitoring the rate of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reduction at 412 nm (Wehrmann et al. 1996). This reaction was performed at 25°C in a solution containing 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.33 mM DTNB, 0.25 mM acetyl-coenzyme A, and 1 mM phosphinothricin (PPT). It was started by the addition of PPT, and activity was determined as described previously (Thompson et al. 1987).

Protein Extraction From Zoysiagrass

Fully expanded young leaves were collected from transgenic zoysiagrass and ground in liquid nitrogen to a fine power. The powder (0.1 g) was resuspended in 200 μl of a buffer solution consisting of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1% PVPP. Extracts were centrifuged at 15,000 rpm for 30 min at 4°C, and the resulting supernatant was used for our SGF assay. The protein concentration of extracts was determined with a BCA protein assay kit (Pierce).

Digestion of PAT with SGF

Simulated gastric fluid was prepared as a solution of 0.084 N HCl and 35 mM NaCl, with the pH adjusted to 2.0 (Thomas et al. 2004). Purified powdered pepsin (Sigma) was added to the SGF based on the units of activity per milligram of protein (approximately 7,000 units of pepsin per milligram of PAT). This protein (100 μl) was incubated with the same volume of preheated SGF—with or without pepsin—for up to 60 min at 37°C. Samples (20 μl) were removed at 0.5, 1, 2, 5, 10, and 60 min after incubation began and were diluted in 20 μl of a stop solution [0.12 M Tris–HCl (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, 4% SDS, 0.09 M NaOH, and 0.04% bromophenol blue], then boiled for 5 min at 100°C. Digestion samples of the protein at the zero time point were prepared by vigorously mixing SGF into the stop solution before adding PAT. Horseradish peroxidase (HRP; Sigma) and chicken ovalbumin (OVA; Sigma) were treated with SGF under the same conditions and included in the analysis for reference (Herouet et al. 2005). These samples were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting. To determine acetyltransferase activity, 10-μl aliquots were removed from the reaction and immediately diluted in 90 μl of ice-cold 1 M Tris–1HCl (pH 8.0) and 20 mM EDTA buffer. They were then stored on ice.

SDS–PAGE and Western Blot Analysis

For each time point, samples were separated by SDS–PAGE in 12% polyacrylamide tricine gels (HRP and OVA) or Tris-glycine gel (PAT), and the gels were stained with 0.2% Coomassie brilliant blue R-250 for 20 min before

being destained with 7.5% acetic acid. To analyze the samples derived from crude protein extracts of transgenic zoysiagrass, we performed Western blot analysis. Samples were separated by SDS–PAGE and transferred onto Hybond-P membranes (GE Healthcare). After blocking with 3% gelatin, the blots were reacted with affinity-purified anti-PAT antibodies (SIGMA P0249) at room temperature for 2 h. The immunoreactive proteins were detected with a Western blotting detection kit (Bio-Rad).

PAT Assay Using Protein Test Strips After Digestion with SGF

We used Trait LL Test Strips (Strategic Diagnostics Inc.) to assay the digestibility of the PAT protein from leaf tissue powder in SGF. These strips detect protein via immuno-chromatography. Antibodies specific to the PAT protein are coupled to a color reagent and incorporated into the lateral flow strip. The test strip displays two red lines if the protein is present. For this assay, our control was the total soluble proteins from transformed zoysiagrass that had been prepared and treated with SGF as described above. Samples from leaf tissues also were collected and ground to fine powder in liquid nitrogen. SGF solutions with pepsin were then added directly to about 0.1 g of that powder. Each sample was incubated at 37°C; the reactions were stopped at 0.25, 0.5, 1, 2, and 5 min after initiation by adding an ice-cold stop solution of 1 M Tris–HCl (pH 8.5) and 20 mM EDTA. The sample for the zero time point was prepared by vigorously mixing SGF with this stop solution before adding the leaf tissue.

Results

Molecular Characterization of GM Herbicide-Tolerant Zoysiagrass

We evaluated the *bar* gene in GM herbicide-tolerant zoysiagrass with genomic DNA blot analysis. Prior to these experiments, the T-DNA region of pGPTV-HB (Lee et al. 1998; Toyama et al. 2003; Bae et al. 2008) had been completely sequenced because knowing the exact DNA sequence is valuable to the molecular characterization and risk assessment of that crop. Our gene probe hybridized to one band in *Hin*dIII and *Xho*I digestion, indicating that GM zoysiagrass has only a copy of *bar* (Fig. 1). This probe also hybridized exactly to the 2.0-kb band that was expected in *Eco*RI digestion.

Using a TAIL-PCR strategy (Liu et al. 1995), we sequenced 926 bp of the right and 644 bp of the left junction sequences of T-DNA (Fig. 2a). Those sequences, searched with bioinformatics tools, showed no homology

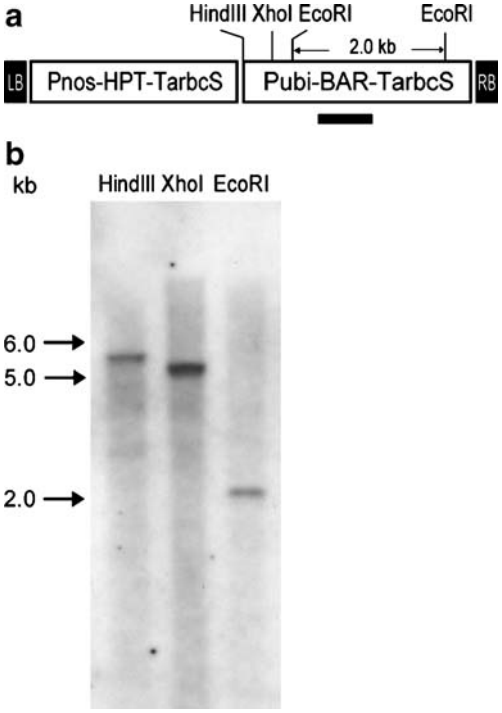

Fig. 1 Gel blot analysis of *bar*. **a** The simplified T-DNA of pGPTV-HB. The 700-bp *bar* DNA amplified by PCR was used as probe. **b** JG21 zoysiagrass genomic DNA (10 μg) was digested with *Hin*dIII, *Xho*I, or *Eco*RI and resolved on 1.0% agarose gel. DNA sizes are indicated by *arrows*

with any from the GenBank sequence database according to BlastN, and the deduced amino acid sequence did not comprise a significant peptide stretch based on our BlastX search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, within each end of the 1,570-bp flanking T-DNA no characteristics for a promoter were found with a scan analysis (http://www.bimas.cit.nih.gov/cgi-bin/molbio/proscan). These results therefore indicate that the T-DNA insertion in our GM zoysiagrass occurred in a chromosomal region that does not encode protein.

We used nested PCR to genotype 23 progenies (T_1 generation) from 7-year-old primary transgenic zoysiagrass plants (Fig. 2b, c). Of these, five were homozygotes, 12 were hemizygotes, and six were of the wild type (WT), i.e., a 1:2:1 ratio of homozygote/hemizygote/WT. As expected, all homozygous and hemizygous plants were herbicide tolerant, and all WT segregates were herbicide sensitive (data not shown). We designated this transformation event Jeju Green 21 (JG21).

Degradation of PAT by SGF Compared Between Total Soluble Proteins and Leaf Tissue Powder

An in vitro pepsin digestion assay was performed for the PAT protein expressed by JG21. Here, 552 bp of the *bar*

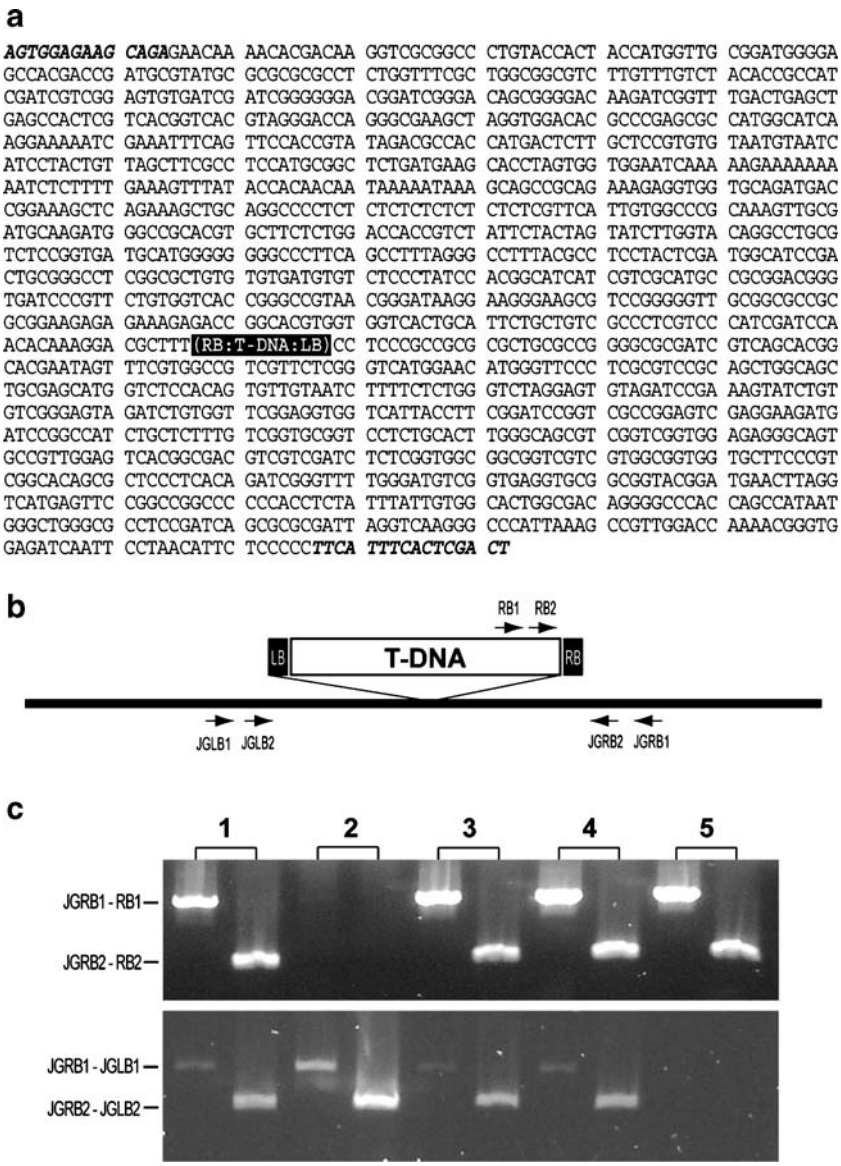

Fig. 2 Molecular analysis of JG21 zoysiagrass. **a** Isolation of DNA sequences flanking right or left border of T-DNA. *Black box* indicates position of T-DNA. *Bold italic letters* at each 5′ and 3′ end coincide with sequences of degenerate primers used in TAIL-PCR. **b** This diagram shows the position of the primers used for genotyping the transgenic plants. *Arrows* identify primers for PCR. **c** Genotyping T-DNA insertion from progenies of primary transgenic plants. Specific amplification of target DNA was confirmed by nested PCR. *Lanes 1*, *3*, and *4* are hemizygotes; *lane 2*, wild-type segregate; *lane 5*, homozygote

gene encoding PAT was amplified from the genomic DNA of JG21 and introduced into *E. coli*. The resultant protein, when purified by affinity chromatography, presented a single band with a molecular weight of about 29 kDa on the reducing SDS–PAGE (Fig. 3c, lanes 1 and 2). When measured with an acetyltransferase assay, specific activity of the purified PAT enzyme was present at 0.61 units. Protein degradation by SGF has historically been monitored with two techniques—SDS–PAGE (Herouet et al. 2005) and an acetyltransferase spectrophotometric standard assay (Wehrmann et al. 1996). HRP (Fig. 3a) and OVA (Fig. 3b) were rapidly and slowly degraded in the SGF, respectively. In agreement with results reported by Herouet et al. (2005), our purified PAT protein was degraded within 30 s after pepsin treatment (Fig. 3c); enzyme activity of that protein then decreased to zero within 30 s (Fig. 3d).

Total soluble proteins from JG21 (Fig. 4a, lane 2) and WT (Fig. 4a, lane 1) zoysiagrass were resolved by SDS–PAGE and visualized by Coomassie brilliant blue staining. Degradation was confirmed by Western blotting and enzyme assays. PAT obtained from zoysiagrass proteins also was degraded within 30 s after pepsin treatment (Fig. 4a, b). However, when pepsin was omitted from the SGF, no significant degradation was observed (Fig. 3a, lane 2; Fig. 4a, lane 3). Nevertheless, enzyme activity decreased rapidly (data not shown), suggesting that the acidic pH of the SGF was sufficient to inactivate the PAT protein.

Traditional in vitro pepsin digestion assays that evaluate the digestibility of pure protein are not adequate for evaluating how complex matrices in foods or raw crop materials can influence digestion stability (Ofori-Anti et al. 2008). Therefore, we used a PAT detection strip kit to

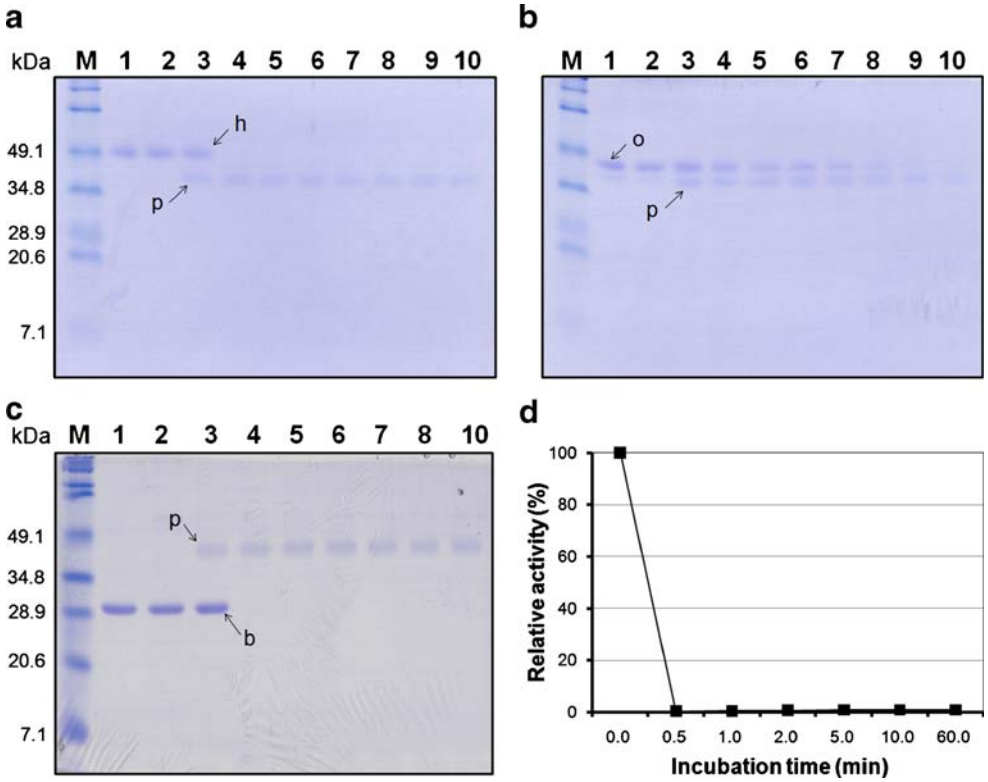

Fig. 3 SDS–PAGE analysis of three proteins (**a** HRP; **b** OVA; **c** PAT) and PAT assay (**d**) of purified protein from *E. coli* after treatment with simulated gastric fluid. Protein was incubated in SGF without [*lanes 1* (0 min) and *2* (60 min)] or with [*lanes 3–10* (0, 0.5, 1, 2, 5, 10, 30, or 60 min)] pepsin. Samples were taken at times indicated. As two controls for SGF assay, HRP (**a**) and OVA (**b**), which rapidly and slowly digested proteins, respectively, were used. Remaining enzyme activity (**d**) was measured as described in "Materials and Methods." *M* protein size marker. Size of protein standard (kDa) is shown at *left*. *p* pepsin; *h* HRP; *o* ovalbumin; *b* PAT

compare stability between leaf tissue powder and total soluble proteins in SGF (Fig. 5). Although PAT in the total soluble proteins was degraded rapidly, in 15 s, after adding pepsin (Fig. 5b), for the powder, complete digestion required at least 5 min (Fig. 5a). This indicated that the leaf powder of transgenic zoysiagrass might have affected the degradation of the PAT protein, a conclusion based on the lack of significant differences in sample amounts, pepsin units, or SGF pH between the two tests.

Discussion

Transformation Event of T-DNA in GM Herbicide-Tolerant Zoysiagrass

GM herbicide-tolerant zoysiagrasses have been generated to contain the *bar* gene (Toyama et al. 2003). Prior to its legal release for commercial purposes, substantial equivalences were established between transgenic and WT plants

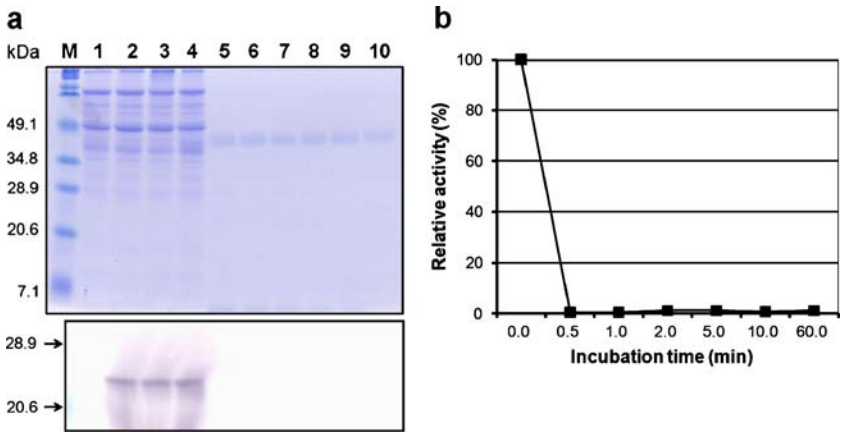

Fig. 4 SDS–PAGE (**a**, upper), Western blot analysis (**a**, lower), and PAT assay (**b**) of total soluble proteins extracted from herbicide-tolerant zoysiagrass after digestion. Total soluble protein was incubated in SGF without [*lanes* 2 (0 min) and *3* (60 min)] or with [*lanes 4–10* (0, 0.5, 1, 2, 5, 10, or 60 min)] pepsin. Samples were taken at times indicated. Remaining enzyme activity (**b**) was measured as described in "Materials and Methods." *Lanes 1*, wild-type zoysiagrass; *M*, protein size marker. Size of protein standard (kDa) is shown at *left*

based on their essentially identical reproduction rates, leaf and seed morphologies, germination rates, chemical composition, and pollen viability/longevity (Bae et al. 2008; Kang et al. 2009). Here, we designated the specific transformation event as JG21. After spraying the T_1 progenies of the first through sixth year vegetatively reproduced primary transgenic plant with Basta, we confirmed that herbicide tolerance segregated in a 3:1 ratio according to Mendelian inheritance. Our genomic DNA blot analysis demonstrated that JG21 carries one copy of the *bar* gene, which has been maintained stably in the T_0 line as a hemizygous genotype for 7 years. When both of its end sequences were isolated and analyzed by BlastN, BlastX, and proscan, the *bar* gene entered a region on the chromosomal DNA that does not encode a protein. Because plant transformation and DNA insertion events themselves can be a mutagen (Jeong et al. 2002; Kim et al. 2003; Latham et al. 2006), selection of a transgenic line that promises substantial equivalence is very important to the commercialization of any GM crop. Insertion of a desired gene into a noncoding region becomes an important qualifying criterion for such line selection because the insertion itself can negatively influence a substantial trait in the targeted crop, e.g., if the gene enters a transcriptionally active sequence region on a chromosome. Our results, however, suggest that JG21 zoysiagrass, which is tolerant to the nonselective Basta, does not cause a serious mutation due to the insertion event.

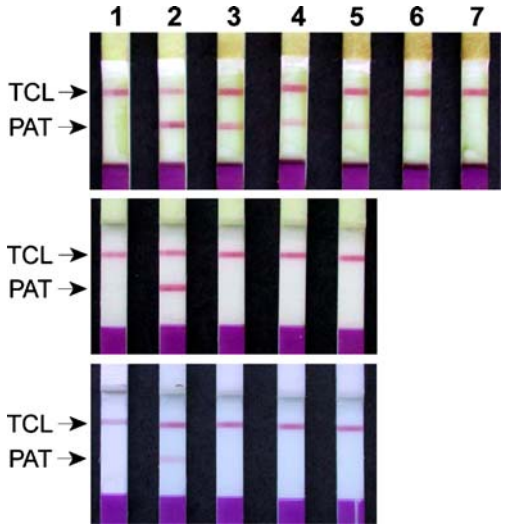

Fig. 5 Protein test strip analyses for PAT proteins (*top*, leaf tissue powder from GM zoysiagrass; *middle*, total soluble protein from GM zoysiagrass; *bottom*, purified PAT protein from *E. coli*) after digestion. Each sample was incubated with SGF for 0, 0.25, 0.5, 1, 2, or 5 min (*lanes 2–7*, respectively). *Lane 1* represents leaf tissue powder from wild-type zoysiagrass (*top*), total soluble protein from wild-type zoysiagrass (*middle*), and distilled water (*bottom*). TCL is test control line

Degradation of the PAT Protein From Leaf Tissue Powder in SGF

Novel genes, such as those for herbicide tolerance or insect resistance, have been transferred into many commercial GM crops, including corn, cotton, soybean, and the canolas (*Brassica napus* L. and *Brassica rapa* L.; http://bch.cbd.int/database/organisms/). Accompanying such development and commercialization, it has become necessary to vise methods that can reasonably assess the potential toxicity or allergenicity of these transgenic products (Sesikeran and Vasanthi 2008). Here, we evaluated the reliability of in vitro pepsin digestion assays to assess the digestibility of the PAT protein. The safety of this protein, as purified from *E. coli*, has been demonstrated in earlier tests. Those investigations have included homology comparisons with known allergens or toxins, searches for N-glycosylation sites, intravenous applications with mice, and determining the susceptibility of PAT to pepsin in SGF (Wehrmann et al. 1996; Herouet et al. 2005; Bae et al. 2008). All of those have shown that PAT itself causes no harm to human or animal food sources. In addition, no differences in structural and functional equivalences have been found between PAT protein produced from *E. coli* and that extracted from transgenic crops (Herouet et al. 2005). Our experiments in particular were focused on the degradation of PAT from both ground leaf tissue powder and purified protein. After pepsin was added to each, PAT from the total soluble proteins degraded rapidly (15 s) while the rate was slower in the powdered samples, not becoming completely digested until 5 min after the test was initiated. However, the standard for defining a protein as having an increased risk of causing a food allergy is that it cannot be digested to 10% or less residual mass by SGF in about 20 min (Ofori-Anti et al. 2008).

The in vitro pepsin digestion assay, which evaluates the susceptibility of purified proteins to SGF, has been adopted as an international guideline for assessing the safety or risk of novel proteins newly produced in GM crops (Thomas et al. 2004). However, researchers have more recently expressed some doubts about the adequacy of this protocol and its objective detection limit (Moreno 2007; Ofori-Anti et al. 2008). One concern is that the matrices in GM crops or food products may influence the digestion stability of specific proteins in SGF. Our experimental results suggest that some matrices from the leaf tissues of GM zoysiagrass may disturb or delay the degradation of a novel protein.

In summary, however, we have confirmed here that herbicide-resistant JG21 carries a single copy of *bar* and that this gene is inserted into a chromosomal region and is not active transcriptionally. In addition, through our examination of traditional and modified in vitro pepsin digestion assays, we can conclude that the PAT protein in

JG21 zoysiagrass can be safely and rapidly digested in the stomachs of humans or livestock.

Acknowledgements We thank Priscilla Licht for critical proofreading of the manuscript and Ok-Cheol Jeong and Hye-Ji Baek for their technical assistance. This work was supported in part by grants from the Bio Green 21 Program (Code 20080401034014) and from the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2009-0094062 and 2009-0094061).

References

Bae TW, Vanjildorj E, Song SY, Nishiguchi S, Yang SS, Song IJ, Chandrasekhar T, Kang TW, Kim JI, Koh YJ, Park SY, Lee J, Lee YE, Ryu KH, Riu KZ, Song PS, Lee HY (2008) Environmental risk assessment of genetically engineered herbicide-tolerant *Zoysia japonica*. J Environ Qual 37:207–218

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci U S A 81:1191–1195

Herouet C, Esdaile DJ, Mallyon BA, Debrutne E, Schulz A, Currier T, Hendrickx K, van der Klis RJ, Rouan D (2005) Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequence that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regul Toxicol Pharmacol 41:134–149

Jeong DH, An S, Kang HG, Moon S, Han JJ, Park S, Lee HS, An K, An G (2002) T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol 130:1636–1644

Jones DD, Maryanski JH (1991) Safety considerations in the evaluation of transgenic plants for human foods. In: Levin MA, Strauss HS (eds) Risk assessment in genetic engineering. McGraw-Hill, New York, pp 64–82

Kang HG, Jang S, Chung JE, Cho YG, An G (1997) Characterization of two rice MADS box genes that control flowering time. Mol Cell 7:559–566

Kang HG, Bae TW, Chung OC, Sun HJ, Lim PO, Lee HY (2009) Evaluation of viability, shedding pattern, and longevity of pollen from genetically modified (GM) herbicide-tolerant and wild-type zoysiagrass (*Zoysia japonica* Steud). J Plant Biol 52:630–634

Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. Plant Mol Biol 52:761–773

Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. J Biomed Biotechnol 2006:1–7

Lee HY, Lee CH, Kim HI, Han WD, Choi JE, Kim JH, Lim YP (1998) Development of bialaphos-tolerant transgenic rice using *Agrobacterium tumefaciens*. Kor J Plant Tiss Cult 25:283–288

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

Moreno FJ (2007) Gastrointestinal digestion of food allergens: effect on their allergenicity. Biomed Pharmacother 61:50–60

Mossinger H, Dietrich W (1998) Activation of hemostasis during cardiopulmonary bypass and pediatric aprotinin dosage. Ann Thorac Surg 65:S45–S50

Nap JP, Metz PL, Escaler M, Conner AJ (2003) The release of genetically modified crops into the environment. Part I. Overview of current status and regulations. Plant J 33:1–18

Ofori-Anti AO, Ariyarathna H, Chen L, Lee HL, Pramod SN, Goodman RE (2008) Establishing objective detection limits for pepsin digestion assay used in the assessment of genetically modified foods. Regul Toxicol Pharmacol 52:94–103

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76

Sambrook JJ, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schmidt JO (1994) Toxicology of venoms from the honeybee genus *Apis*. Toxicon 33:917–927

Sesikeran B, Vasanthi S (2008) Constantly evolving safety assessment protocols for GM foods. Asia Pac J Clin Nutr 17:241–244

Sjoblad R, McClintock JT, Engler R (1992) Toxicological considerations for protein components of biological pesticide products. Regul Toxicol Pharmacol 15:3–9

Teng PP (2008) An Asian perspective on GMO and biotechnology issues. Asia Pac J Clin Nutr 17(Suppl 1):237–240

Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, Fu TJ, Glatt CM, Hadfield N, Hatzos C, Hefle SL, Heylings JR, Goodman RE, Henry B, Herouet C, Holsapple M, Laddics GS, Landry TD, MacIntosh SC, Rice EA, Privalle LS, Steiner HY, Teshima R, van Ree R, Woolhiser M, Zawodny J (2004) A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul Toxicol Pharmacol 39:87–98

Thompson CH, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J 6:2519–2523

Toyama K, Bae CH, Kang JG, Lim YP, Adachi T, Riu KZ, Song PS, Lee HY (2003) Production of herbicide-tolerant zoysiagrass by *Agrobacterium*-mediated transformation. Mol Cells 16:19–27

Wehrmann A, Vliet AV, Opsomer C, Botterman J, Schulz A (1996) The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nat Biotechnol 14:1274–1278