Genetic characterization of a novel *Tib*-derived variant of soybean Kunitz trypsin inhibitor detected in wild soybean (*Glycine soja*)

Ke-Jing Wang, Tetsuro Yamashita, Masao Watanabe, and Yoshihito Takahata

Abstract: A novel variant of soybean Kunitz trypsin inhibitor (SKTI) was detected in 530 lines of wild soybean (*Glycine soja*). This variant showed an intermediate electrophoretic mobility between the *Tia* and *Tic* types. In isoelectric focusing polyacrylamide gel electrophoresis gels containing urea, this variant had a similar isoelectric point as that of *Tia*. The genetic analysis of SKTI bands in F_2 seeds from crosses of the new variant type with *Tia* or *Tic* type showed that this variant type is controlled by a codominant allele at the SKTI locus. We propose the genetic symbol *Tif* for this novel variant. When the nucleotide sequence of the *Tif* gene was compared with those of other types of SKTI genes (*Tia*, *Tib*, and *Tic*), the sequence of *Tif* was identical to that of *Tib* with the exception of one A→G transitional mutation occurring at position 676 of *Tif*. This mutation resulted in an amino acid change from Lys to Glu at the 178 residue. These results suggest that this variant is derived from *Tib* through a point mutation. In addition, we settled an inconsistency in the number of amino acid differences between *Tia* and *Tib* (eight or nine). Analysis of nucleotide and amino acid sequences revealed that *Tib* was different from *Tia* by nine amino acids.

Key words: soybean Kunitz trypsin inhibitor, polymorphism, gene sequence, soybean, wild soybean.

Résumé : Un nouveau variant de l'inhibiteur de trypsine de type Kunitz chez le soja (SKTI) a été détecté parmi 530 lignées de soja sauvage (*Glycine soja*). Ce variant présente une mobilité électrophorétique intermédiaire entre les types *Tia* et *Tic*. Sur des gels PAGE à focalisation isoélectrique contenant de l'urée, ce variant montre un point isoélectrique semblable à celui de *Tia*. Une analyse génétique des bandes SKTI au sein d'une population de graines F_2 issues du croisement entre le nouveau variant et les types *Tia* ou *Tic* a révélé que ce variant est contrôlé par un allèle codominant au locus SKTI. Les auteurs proposent le symbole *Tif* pour ce nouveau variant. En comparant la séquence nucléotidique du gène *Tif* à celle des autres gènes SKTI (*Tia*, *Tib* et *Tic*), il a été observé qu'une seule transition A→G, à la position 676 du gène *Tif*, distingue ce dernier de la séquence du gène *Tib*. Cette mutation entraîne un changement d'acide aminé (Lys → Glu) au résidu 178. Ces résultats suggèrent que ce variant est survenu suite à une mutation ponctuelle dans le gène *Tib*. Des plus, les auteurs ont levé l'incertitude quant au nombre de différences qui distinguent *Tia* de *Tib* (huit ou neuf). L'analyse des séquences nucléotidiques et peptidiques a révélé que *Tib* diffère de *Tia* au niveau de neuf acides aminés.

Mots clés : inhibiteur de la trypsine de type Kunitz chez le soja, polymorphisme, séquence nucléotidique, soja, soja sauvage.

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Introduction

Proteinase inhibitors are extensively contained in many plants and are considered to be involved in the control of endogenous proteinases, storage proteins, and defence against insect and microorganism damage (Ryan 1981; Richardson 1977; Johnson et al. 1989; Marchetti et al. 2000). The total seed protein of soybean (*Glycine max*) contains about 6% proteinase inhibitors. These inhibitors have been identified as belonging to two major groups, the Kunitz trypsin inhibitor (Kunitz 1945) and the Bowman–Birk trypsin inhibitor (Bowman 1946; Birk 1961). Of these, the soybean Kunitz trypsin inhibitor (SKTI) has been found to have six electrophoretic distinguishable forms: *Tia* and *Tib* (Singh et al.

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K.-J. Wang. Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan, and Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences, Beijing 100081, China.
T. Yamashita, M. Watanabe, and Y. Takahata.[1] Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan.

[1]Corresponding author (e-mail: ytakahata@iwate-u.ac.jp).

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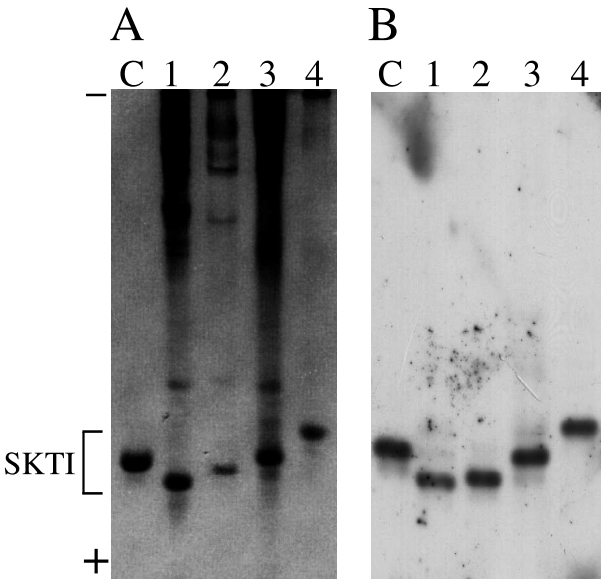
Fig. 1. Identification of a novel variant of SKTI proteins by (A) a Davis system of PAGE and (B) Western blot analysis. Lane C, control of commercial SKTI (*Tia*) from Sigma; lane 1, *Tic* type ('Raiden'); lane 2, a novel variant of SKTI of *G. soja*; lane 3, *Tia* ('Rikuu No.27'); lane 4, *Tib* ('Tachisuzunari').



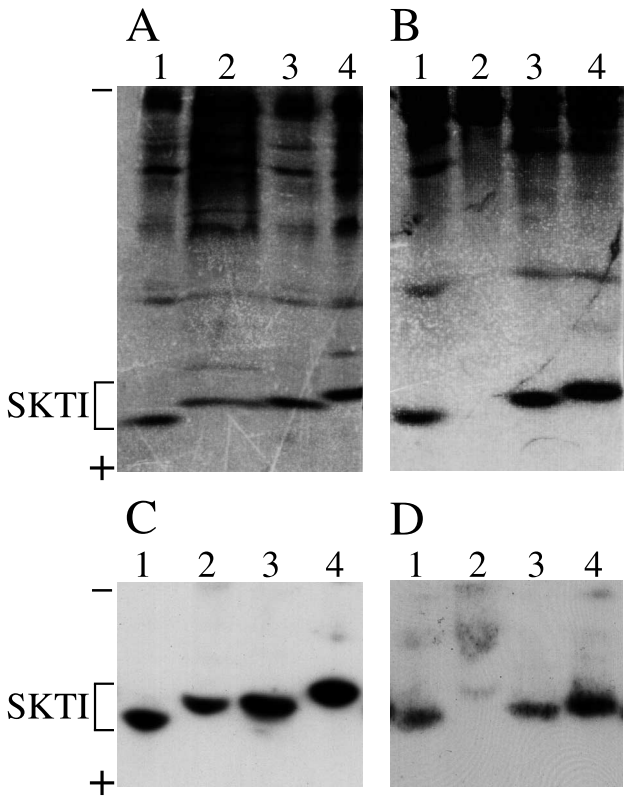
1969), *Tic* (Hymowitz 1973), *Tid* (Zhao and Wang 1992), *Tie* (Wang et al. 1996, 2001), and *ti* (null type) (Orf and Hymowitz 1979). These types are controlled by codominant multiple alleles at a single locus (Singh et al. 1969; Hymowitz and Hadley 1972; Orf and Hymowitz 1977; Wang et al. 2001). The polymorphism of SKTI has been widely used for the research of genetic diversity, dissemination, and origin of soybean (Hymowitz and Kaizuma 1979; Kaizuma et al. 1980; Yu and Kiang 1993; Xu et al. 1985).

Kaizuma et al. (1980) considered that the *Tia* type is the prototype from which *Tib* and *Tic* were derived. Studies of amino acid and nucleotide sequences revealed that *Tic*, *Tid*, and *Tie* each were originally generated from the prototype *Tia* by a single amino acid substitution (Kim et al. 1985; Jofuku et al. 1989; Song et al. 1993; Xin et al. 1999; Wang et al. 2001). The *Tib* type has also been considered to be a secondary ancient type that arose from *Tia* before the domestication of cultivated soybean from wild soybean (*Glycine soja*) (Kaizuma et al. 1980). If *Tib* is a second prototype, it is probable that some variant types derived from *Tib* are still in existence. However, so far, such variants have not been found.

Since Kim et al. (1985) reported a comparative study of the amino acid sequences of SKTI, it has been believed that *Tib* differed from *Tia* by eight amino acid residues. In contrast, a study of nucleotide sequences showed a difference of nine amino acids between them (Song et al. 1993). Therefore, a reanalysis is needed to account for this inconsistency. There are such large sequence differences between *Tia* and *Tib*, but no intermediate types between them have been found in either cultivated or wild soybean.

In the present report, we describe detection and genetic characterization of a novel polymorphic variant (designated as *Tif*) of SKTI derived from the *Tib* type in wild soybean

Fig. 2. Identification of a novel variant of SKTI proteins by (A and B) IEF–PAGE and (C and D) Western blot analysis (A and C) containing 8M urea and (B and D) without urea. Lane 1, *Tic* type ('Raiden'); lane 2, a novel variant of SKTI of *G. soja*; lane 3, *Tia* ('Rikuu No. 27'); lane 4, *Tib* ('Tachisuzunari').



and settle the discrepancy in the number of amino acids that are different between *Tia* and *Tib*.

Materials and methods

Plant materials

In this investigation, 530 wild soybean (*G. soja*) lines collected in China were used to analyze the polymorphism of the SKTI proteins by electrophoresis. Soybean (*G. max*) cultivars or lines 'Rikuu No. 27' (*Tia*), 'MG-3' (*Tia*), 'Odate No. 1' (*Tib*), 'Norin No. 2' (*Tib*), 'Tachisuzunari' (*Tib*), 'Raiden' (*Tic*), and 'L-188-21' (*Tic*) and a wild soybean line 74 (*Tib*) collected in China were used as materials of standard SKTI proteins.

Electrophoretic detection

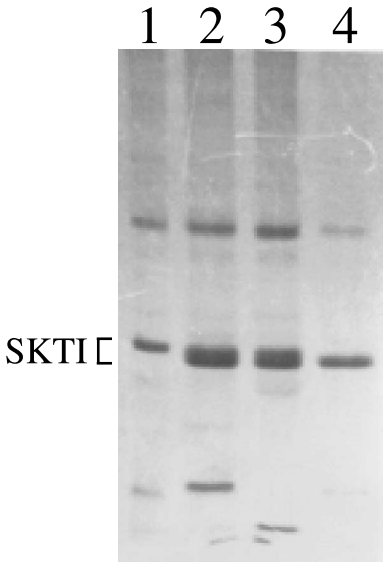
Extraction of SKTI proteins from seeds was carried out according to Hymowitz and Hadley (1972). The SKTI proteins were analyzed by a Davis system of polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF)–PAGE as described previously (Wang et al. 1996) with minor modifications. IEF–PAGE was performed using a gel containing 1.2% pharmalyte (pH 4–6.5) and 0.4% pharmalyte (pH 3–10) with or without 8 M urea.

After electrophoresis, the proteins were electroblotted onto a Immobilon-P membrane (Millipore) for Western blot analysis. SKTI proteins were detected using anti-trypsine in-

Table 1. SKTI band segregation of F_2 seeds from the crosses of a new variant (*Tif*) with *Tia* and *Tic* types.

Cross	Population (F_1 plant)	No. of F_2 seeds examined	Segregation of SKTI bands			Expected ratio	χ^2	Probability
			Tia	*Tia/Tif*	*Tif*			
Tia ('MG-3') × *Tif*	1	100	29	46	25	1:2:1	0.96	$0.5 < p < 0.7$
	2	100	26	44	30	1:2:1	1.76	$0.3 < p < 0.5$
			Tic	*Tic/Tif*	*Tif*			
Tic ('L-188-21') × *Tif*	1	100	25	48	27	1:2:1	0.24	$0.7 < p < 0.9$
	2	100	27	43	30	1:2:1	2.14	$0.3 < p < 0.5$
	3	100	24	53	23	1:2:1	0.38	$0.7 < p < 0.9$

Fig. 3. Segregation of the *Tif* variant in F_2 derived from F_1 between *Tif* and *Tic* ('Raiden'). Lane 1, *Tif* type; lanes 2 and 3, hetero type having both *Tif* and *Tic*; lane 4, *Tic* type.



hibitor (soybean) rabbit antiserum (Rockland) and ECL Western Blotting Detection Regents Kit (Amersham Biosciences).

Genetic analysis

The new variant line of SKTI (*Tif*) proteins found in this study was crossed with the *G. max* 'MG-3' (*Tia*) and 'L-188-21' (*Tic*) lines in a growth chamber. The F_1 plants of these crosses were grown in an experimental field to obtain F_2 seeds, and SKTI of the F_2 seeds was examined electrophoretically.

N-terminal amino acid sequence analysis

SKTI proteins were separated by IEF–PAGE and electrotransferred to the polyvinylidene difluoride membrane, and the N-terminal amino acid sequences of the blotted proteins were determined using a protein sequencer (PPSQ-21, SHIMADZU).

Gene sequence analysis

Total DNA was extracted from a single seed according to McDonald et al. (1994), Tian et al. (2000), and Wu et al. (2001) with minor modifications. The SKTI gene was amplified by polymerase chain reaction using a set of two primers (forward: 5′-TAGTCCCGATTCTCCCAACA-3′, reverse: 5′-AGTACTCTCACACTTGTGTC-3′) (Wang et al. 2001). After the amplified DNA was cloned using a TA Cloning Kit (Invitrogen) according to Wang et al. (2001), it was sequenced with an ABI Prism 310 sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Two independent clones from each of the two seeds were analyzed in each line.

Results and discussion

Detection of a novel SKTI type (*Tif*) in wild soybean

Almost all SKTI electrophoretic mobility types of the 530 wild soybean showed *Tia* (85%) and *Tib* (15%) types, except for one line. This variant line (line 33) showed a novel SKTI electrophoretic mobility that is intermediate between those of *Tia* and *Tic* (Fig. 1). The variant was also compared with three standard SKTI types (*Tia*, *Tib*, and *Tic*) using IEF–PAGE. The different banding patterns of these types in IEF–PAGE gel are shown in Fig. 2. In the presence of 8 M urea, this variant was definitely distinguished from *Tib* and *Tic* by its isoelectric point, having almost a similar isoelectric point as that of *Tia* (Figs. 2A and 2C). This variant has a feature different from other types of *Tia*, *Tib*, and *Tic* in the IEF system. This variant could not be recognized in the IEF gel without urea because of its disappearing band (Figs. 2B and 2D, lane 2). The exact reason why the band of the variant disappeared in the IEF gel without urea is unknown. One possibility is that the novel SKTI may shape the aggregate form after proteins are extracted. However, to elucidate the mechanism of this phenomenon, further investigation is needed.

To investigate the genetic stability of the variant, the variant plant was grown and the selfed seeds were harvested. The electrophoretic results of the SKTI proteins from the selfed seeds showed that the offspring of the variant had the same mobility band, suggesting that the SKTI type of this variant was an inheritable trait (data not shown). We designated *Tif* as the genetic symbol for this novel variant type. Two populations of F_2 seeds obtained from two F_1s between *Tia* ('MG-3') and *Tif* variant and three populations of F_2 seeds obtained from three F_1s between *Tic* ('L-188-21') and *Tif* were individually analyzed by PAGE for the segregation ratio of the new SKTI type. The genetic segregation ratio of the F_2 seeds in the two crosses showed an acceptable fit to a 1:2:1 ratio of the *Tia* (or *Tic*) band to both the *Tia* (or *Tic*) and the *Tif* bands to the *Tif* band (Table 1; Fig. 3). These re-

Fig. 4. Nucleotide and deduced amino acid sequences of the SKTI genes of *Tia* ('Rikuu No. 27') (DDBJ accession No. AB112031), *Tic* ('Raiden') (DDBJ accession No. AB112033), *Tib* ('Odate No. 1', 'Tachisuzunari', 'Norin No. 2', and *G. soja* line 74) (DDBJ accession No. AB112032), and *Tif* (a novel variant of *G. soja*) (DDBJ accession No. AB112034). Dashes in sequences indicate identical nucleotides and amino acids. The box shows the single amino acid of *Tif* that differed from *Tib.* Horizontal arrows show the positions of primers used to amplify the sequences. The mature SKTI proteins begin at D* and end at L*.

Table 2. Comparison of the beginning 14 amino acid sequences at the N terminus among different SKTI types.

		Amino acid sequence													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sigma production[a]	*Tia*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Glu	Asn	Gly
'Rikuu No. 27'	*Tia*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Glu	Asn	Gly
'Norin No. 2'[a]	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Ser	Asn	Gly
'Paldal'[b]	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly
'Norin No. 2'	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly
'Odate No. 1'	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly
'Tachisuzunari'	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly
G. soja line 74	*Tib*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly
G. soja	*Tif*	Asp	Phe	Val	Leu	Asp	Asn	Glu	Gly	Asn	Pro	Leu	Asp	Ser	Gly

Note: The sequences were determined for the protein bands in an IEF–PAGE gel. Underscoring indicates the correct amino acid sequences at residues 12 and 13 in *Tib*.

[a]Sequences quoted from the results reported by Kim et al. (1985).

[b]Sequences deduced from the nucleotide sequences reported by Song et al. (1993).

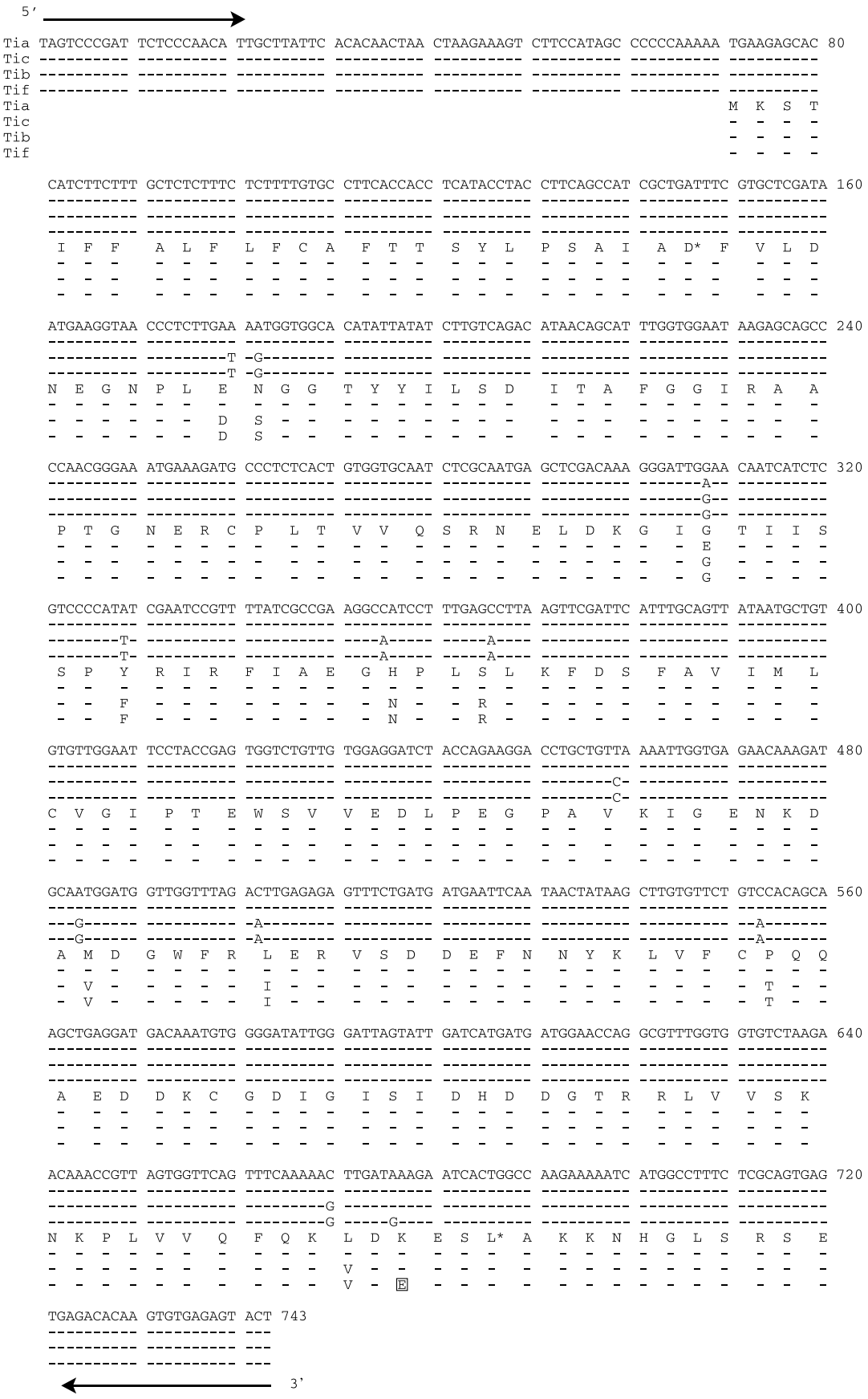
sults demonstrate that *Tif* is a genetically new codominant allele at the SKTI locus.

Nucleotide sequence analysis indicated that all of the amplified fragments had an identical length of 743 bp. The sequences contained an open reading frame of 651 bp encoding 271 amino acids, which consisted of 181 amino acids of a mature SKTI, signal regions of 25 amino acids at the N terminus, and 11 amino acids at the C terminus (Fig. 4). Such extra amino acids at the N terminus and C terminus were reported by Song et al. (1993) and Wang et al. (2001). The sequences of *Tia* and *Tib* were identified with those reported by Song et al. (1993). Comparing the sequence of the *Tif* gene with other types of SKTI, the sequences of *Tif* and *Tib* were identical except for one A→G transitional mutation that occurred at position 676 of *Tif* (Fig. 4). This mutation resulted in an amino acid change from Lys to Glu at the 178 residue. To date, the known SKTI types, such as *Tic* (Kim et al. 1985), *Tid* (Xin et al. 1999), and *Tie* (Wang et al. 2001), have been shown to differ from *Tia* in a single amino acid residue, suggesting that these SKTI types originated from the *Tia* type by point mutation. Our results indicate that the novel SKTI type of *Tif* originated from *Tib.* This was supported by the existence of the same silent mutation at position 459 in both *Tib* and *Tif* (Fig. 4). Namely, the sequence of the *Tib* gene differed from *Tia* and *Tic* by a T→C mutation at position 459, which does not cause amino acid change (*Tia* and *Tic*: GTT, *Tib*: GTC). In common with *Tib*, the *Tif* gene had the identical mutation at the same position.

Comparisons of amino acids between *Tia* and *Tib*

There is inconsistency in the number of amino acid differences between *Tia* and *Tib* SKTI proteins. Kim et al. (1985) reported that the *Tib* type differs from *Tia* by the following eight amino acid residues based on amino acid sequence analysis: Ser (12), Phe (62), Asn (71), Arg (74), Val (114), Ile (120), Thr (137), and Val (176); in contrast, Song et al. (1993) demonstrated that *Tib* differed from *Tia* by the following nine amino acids based on nucleotide sequence analysis: Asp (12), Ser (13), Phe (62), Asn (71), Arg (74), Val (114), Ile (120), Thr (137), and Val (176). The difference between them is present at the 12th and 13th amino acid residues of the mature SKTI protein: Ser (12) – Asn (13) ('Norin No. 2'; Kim et al. 1985) and Asp (12) – Ser (13) ('Paldal'; Song et al. 1993). The possibility remained that such disagreement may be due to the difference in cultivars used. We analyzed the nucleotide sequences of the SKTI genes in four kinds of *Tib* materials ('Norin No. 2', 'Odate No. 1', 'Tachisuzunari', and *G. soja* line 74) and one *Tia* ('Rikuu No. 27'). Of these *Tib* materials analyzed, 'Norin No. 2' is the same cultivar as the one used by Kim et al. (1985). Our results showed that all four *Tib* materials had the same nucleotide sequences, and their deduced amino acids 12 and 13 are Asp–Ser rather than Ser–Asn, suggesting that there were nine amino acid differences between *Tia* and *Tib* and not eight (Fig. 4). This is supported by the results from the sequence analysis of amino acids at the N terminus of the mature SKTI proteins, which revealed that two amino acids were different between *Tib* and *Tia* at this region (Table 2). These results support those of Song et al. (1993) rather than those of Kim et al. (1985). It is thus concluded that *Tib* is different from *Tia* by nine amino acid residues.

The nucleotide sequence of the *Tic* gene ('Raiden'), which was first determined in this study, is identical to the sequence of *Tia* except for one G→A transition occurring at position 308 of *Tic* (Fig. 4). This mutation results in an



amino acid change from Gly to Glu at the 55 residue. This result coincides with the amino acid sequence of *Tic* ('Raiden') revealed by Kim et al. (1985).

In this study, we discovered a novel *Tib*-derived *Tif* variant that is one of the multiple alleles at the SKTI locus. This is the first report showing a variant of SKTI originating from *Tib*. In addition, we determined a sequence difference in nine amino acid residues between *Tia* and *Tib*. Thus far, of several SKTI forms, *Tia*, *Tib*, and *Tic* have been detected in both wild and cultivated soybean, *Tid* was found in cultivated soybean (Zhao and Wang 1992), and *Tie* was observed in wild soybean (Wang et al. 1996, 2001). Since *Tic*, *Tid*, and *Tie* each differ from *Tia* by one amino acid, these types are considered to originate from prototype *Tia* by a point mutation. The *Tif* variant detected in this study differs from *Tib* by one amino acid, showing a novel variant that originated from *Tib*. Kaizuma et al. (1980) considered that the *Tia* type is the prototype from which *Tib* and *Tic* were derived and that differentiation of *Tib* from *Tia* occurred much earlier than that of *Tic* from *Tia*. Our results support their idea that *Tib* is a secondary ancient type of SKTI. On the other hand, there are large differences in nine amino acid residues between *Tia* and *Tib*. Thus far, no intermediate type between them has been found in either soybean or wild soybean. A search of such an intermediate variant is currently being carried out.

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