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Entity evidence for differentiation between *Tia* and *Tib* types of soybean Kunitz trypsin inhibitor: detection of a novel transitional variant type between *Tia* and *Tib* in wild soybean (*Glycine soja* Sieb. & Zucc.)

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Abstract Soybean Kunit trypsin inhibitor (SKTI) has several polymorphic types. Of these SKTI, there are large differences of nine amino acid substitutions between *Tia* and *Tib*. So far no transitional type between them has been found. A novel transitional intermediate variant between *Tia* and *Tib* was detected in 11 lines from 720 Japanese wild soybeans (*Glycine soja* Sieb. & Zucc.). This variant showed identical electrophoretic mobility to *Tib* in the Davis system polyacrylamide gel electrophoresis (PAGE), but higher electric points than other SKTI proteins (*Tia, Tib, Tic*) in isoelectric focusing PAGE. The genetic analysis of SKTI in F_2 seeds from a cross between the novel variant type and *Tib* showed that this variant type is inherited as codominant alleles in a multiple allelic system at an SKTI locus. This variant also showed inhibitory activity to trypsin. We propose the genetic symbol Tib^{i5} for this novel variant. The sequence analysis of Tib^{i5} revealed that six nucleotides were different between Tib^{i5} and *Tia*, and the nucleotides of these mutated positions were identical to *Tib*. This causes substitution of five amino acids at the residue position 62 (Tyr → Phe), 74 (Ser → Arg), 114 (Met → Val), 120 (Leu → Ile) and 137 (Pro → Thr). These substitutive amino acids are completely in accord with the amino acids of *Tib*, showing that Tib^{i5} is an intermediate between *Tia* and *Tib* types. Tib^{i5} type is widely distributed throughout seven separate areas from northeast to southwest Japan with a 1.5% frequency of total materials examined. This indicated that Tib^{i5} type did not originate from a recent mutation event, but had spread in wild soybean from ancient times.

Keywords Kunitz trypsin inhibitor · Polymorphism · Gene sequence · Protein evolution · *Glycine soja* · Soybean

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Introduction

Soybean [*Glycine max* (L.) Merr.] is considered to be domesticated from wild soybean (*G. soja* Sieb. & Zucc.) distributed throughout East Asia (Fukuda 1933; Hymowitz and Singh 1987). These two species have an identical genome (GG) and are classified in the subgenus *Soja* (Moench) F.J. Herm. of the genus *Glycine* (Singh and Hymowitz 1988). The seeds of the subgenus *Soja* contain trypsin protinase inhibitors at a high concentration (about 6% of total proteins). They can be divided into two major groups, the Kunitz trypsin inhibitor (Kunitz 1945) and the Bowman–Birk Inhibitor (Bowman 1946; Birk 1961). Since Singh et al. (1969) identified the polymorphism of the soybean Kunitz trypsin inhibitor (SKTI) such as *Tia* and *Tib*, five polymorphisms of the SKTI have been found: *Tic* (Hymowitz 1973), *ti* (null type) (Orf and Hymowitz 1979), *Tid* (Zhao and Wang 1992), *Tie* (Wang et al. 2001) and *Tif* (Wang et al. 2004). These polymorphisms are controlled by codominant multiple alleles at a single locus (Hymowitz and Hadley 1972; Orf and Hymowitz 1979; Wang et al. 2001, 2004).

The polymorphism of SKTI has often been used as an index for investigating the genetic diversity, geographical diversification, origin of soybean and phylogenetic relationship among species of *Glycine* (Hu and Wang 1985; Fujita et al. 1997; Yu and Kiang 1993; Hymowitz and Kaizuma 1979; Kaizuma et al. 1980;

Kiang et al. 1992; Wang et al. 1998). *Tia* type is reported to be a prototype from which *Tib* and *Tic* were derived (Kaizuma et al. 1980).

Studies of amino acid and nucleotide sequences of polymorphic variants of SKTI have revealed that there is a large sequence difference in nine amino acid residues between *Tia* and *Tib* (Song et al. 1993; Wang et al. 2004); each *Tic*, *Tid* and *Tie* differ by only one amino acid from *Tia* type (Kim et al. 1985; Xin et al. 1999; Wang et al. 2001) and *Tif* differs by one amino acid from *Tib* type (Wang et al. 2004). Such a large difference of nine amino acid substitutions between *Tia* and *Tib* makes it difficult to conceive that *Tib* type had been differentiated from *Tia* through a single mutational event. Therefore, some transitional forms with one or more amino acid residue difference(s) should exist in the subgenus *Soja*. However, such transitional intermediate types have not yet been found, which has caused a gap to remain in terms of the genetic differentiation between *Tia* and *Tib* types.

In this paper we report detection and genetic and physiological characterization of a novel transitional intermediate type (designated as Tib^{i5}) between *Tia* and *Tib* of SKTI in wild soybean. In addition, the evolution of SKTI proteins is discussed.

Materials and methods

Plant materials

For detection of polymorphism of the SKTI protein, 720 lines of *G. soja*, which are being preserved at Soybean Breeding Lab, National Agricultural Research Centre for Tohoku Region (NARCT) and Plant Breeding Lab., Iwate University, were used. Soybean cultivars, Rikuu No. 27 (*Tia*), Tachisuzunari (*Tib*) and Raiden (*Tic*) were used as standard types of SKTI.

Detection of SKTI by electrophoreses

SKTI proteins were extracted from seeds according to Hymowitz and Hadley (1972). The SKTI proteins were analyzed by a Davis system polyacrylamide gel electrophoresis (PAGE) and isoelectrofocusing (IEF) PAGE using 9 cm x 9 cm gels as described by Wang et al. (1996). Western bolt analysis of the SKTI was carried out according to Wang et al. (2004). After electrophoresis, the proteins were electroblotted onto a PVDF membrane. SKTI proteins were detected using anti-trypsin inhibitor (soybean) rabbit antiserum (Rockland) and an ECL Western Blotting Detection Reagents Kit (Amersham Biosciences).

Trypsin inhibitor activity assay

SKTI was purified from crude seed proteins of L24 line (Tib^{i5}) and 'Tachisuzunari' (*Tib*) by FPLC (Pharmacia Biotech). The purified SKTIs were confirmed by Davis system PAGE. Determination of inhibitory activity was carried out according to Kim et al. (1985). An aliquot of the inhibitor solution was mixed with 14.6 μg of trypsin, and the mixture was adjusted to 2.5 ml with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$. Two milliliters of the mixture was pipetted into a cuvette, and 0.1 ml of 10 mM BAEE solution in the same buffer was added. The change in absorbance at 253 nm was measured.

Genetic analysis

The L24 line, which is one of the new variant lines of SKTI protein (Tib^{i5}) found in this study, was crossed with the *G. max* cv. Tachisuzunari (*Tib*). F_1 plants of this hybridization were grown to obtain F_2 seeds, and SKTI proteins of F_2 seeds were investigated electrophoretically.

Nucleotide sequence analysis

Gene sequence analysis was carried out according to Wang et al. (2001) with minor modification. Total DNA was extracted from a single seed in wild soybean and 30 mg meal pared from a seed in cultivated soybean using a Dneasy Plant Mini Kit (Qiagen Inc.). The SKTI gene was amplified by PCR using a set of two primers (forward: 5′-TAGTCCCGATTCTCCCAACA-3′, reverse: 5′-AGTACTCTCACACTTGTGTC-3′) designed on the basis of DNA sequences of *Kti3* (= *Tia*) reported by Jofuku et al. (1989). After the amplified DNA was cloned using a TA Cloning Kit (Invitrogen), it was sequenced with an ABI Prism 310 Genetic Analyzer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Results

Identifying a novel electrophoretic type (Tib^{i5}) of SKTI

SKTI proteins of the wild soybean lines were first analyzed by Davis system PAGE, and SKTI electrophoretic types of 720 wild soybean lines showed two SKTI types, *Tia* and *Tib*, with a frequency of 86 and 14%, respectively. IEF-PAGE analysis showed a novel SKTI electrophoretic mobility in 11 of 101 lines showing *Tib* type in Davis system PAGE. Though this variant type had the same mobility as *Tib* in Davis system PAGE, it had a different electric point from *Tib* in IEF-PAGE (Figs. 1, 2). The electric point of this variant was higher than those of the standard types of SKTI (*Tia, Tib* and *Tic*). Eleven lines showing the new variant type designated at Tib^{i5} were collected from different areas of Japan from the northeast area (31°46′ N) to the southeast area (39°32′ N) (Table 1). Of these 11 lines, three, S194, S204

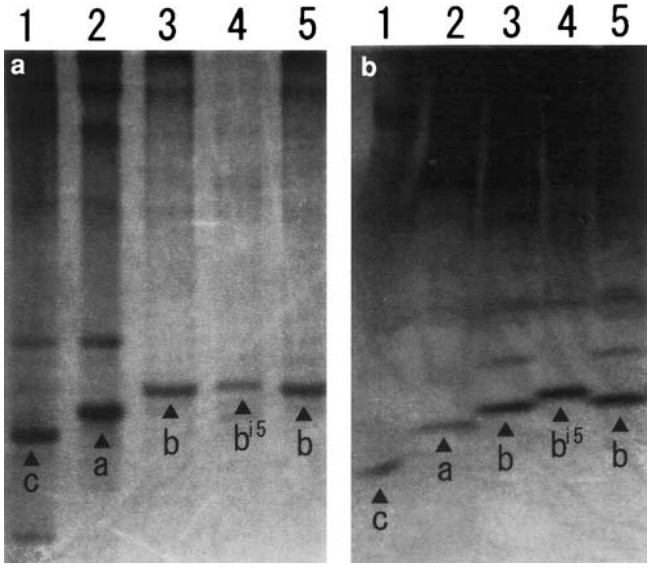

Fig. 1 Electrophoretic profiles of a novel variant of SKTI proteins by a Davis system of PAGE (**a**) and a isoelectrofocusing PAGE (**b**). *Lane 1 G. max* 'Raiden' (*Tic* type), *lane 2* 'Rikuu No. 27' (*Tia*), *lanes 3 and 5* 'Tachisuzunari' (*Tib*), *lane 4 G. soja* line S194 (a novel variant *Tib*^{i5})

and S205, were collected from separate nearby populations along a river, and N249 and N250 were collected from other populations at the same site. The other five lines were each distributed throughout different areas.

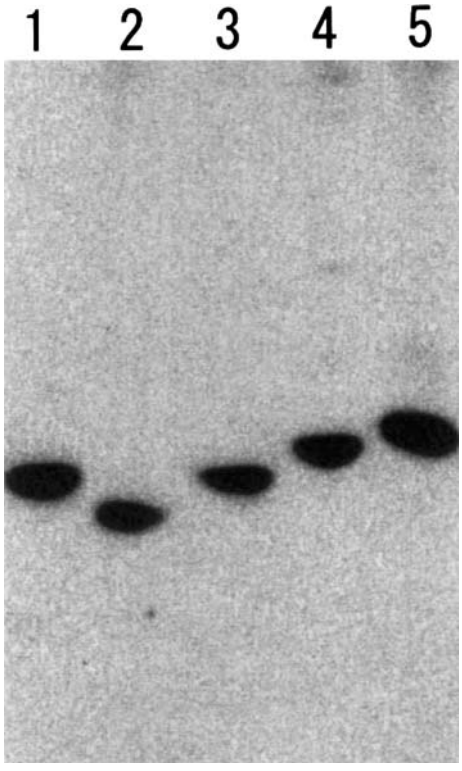

Fig. 2 Identification of SKTI by western blot analysis. *Lane 1* commercial SKTI (*Tia*) by Sigma, *lane 2 G. max* 'Raiden' (*Tic*), *lane 3* 'Rikuu No. 27' (*Tia*), *lane 4* 'Tachisuzunari' (*Tib*), *lane 5 G. soja* line S194 (a novel variant *Tib*^{i5})

Trypsin inhibitory activity of *Tib*^{i5} protein

To confirm whether the *Tib*^{i5} protein has trypsin inhibitory activity and to understand its inhibitory feature, we compared the inhibitory activity of *Tib*^{i5} and *Tib* types. As shown in Fig. 3, the activity of trypsin was inhibited by an increase in concentration of *Tib*^{i5} SKTI. The inhibitory activity of *Tib*^{i5} was similar to that of *Tib*.

Genetic analysis of *Tib*^{i5} type

The cross of Tachisuzunari (*Tib*) × *G. soja* L24 (*Tib*^{i5}) was carried out for analysis of the inheritance of the *Tib*^{i5} variant band. The electrophoretic results of the SKTI proteins from F_1 seeds showed that they had a single band in Davis system PAGE and two bands in IEF-PAGE which are derived from parents as expected (data not shown). F_2 seeds harvested from F_1 plants were individually analyzed by both Davis system PAGE and IEF-PAGE to clarify the segregation ratio of the novel variant type. F_2 seeds segregated in an expected ratio of 1:2:1 for the *Tib* band to both the *Tib* and the *Tib*^{i5} bands to the *Tib*^{i5} band (Table 2, Fig. 4).

Nucleotide sequence of the *Tib*^{i5} gene

All 11 *Tib*^{i5} variant lines were used to determine the nucleotide sequences of the gene. The analysis indicated that all fragments from the 11 lines had identical sequences and contained an open reading frame of 651 bp encoding 271 amino acids, which is the same length as standard types of SKTI such as *Tia*, *Tib* and *Tic*. Comparing the nucleotide sequence of *Tib*^{i5} with standard types of SKTI, this gene had an intermediate trait between *Tia* and *Tib* (Fig. 5). When the sequences of

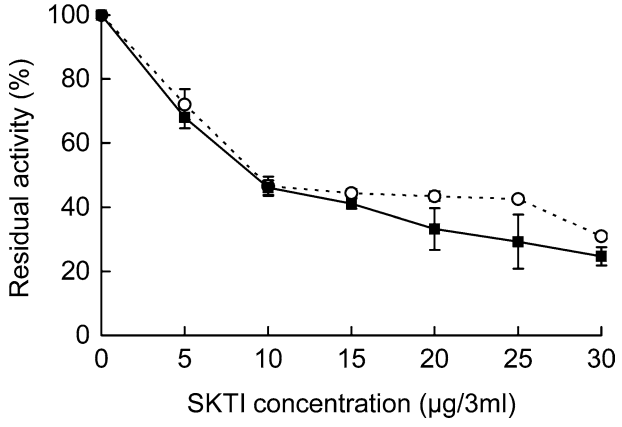

Fig. 3 A comparison of trypsin inhibitor activity between *Tib* and *Tib*^{i5} proteins. A fixed amount (30 μg) of vovine trypsin was mixed with increasing amounts (μg) of the inhibitor proteins. The residual trypsin activity in the mixture was determined by measuring the absorbance under 253 nm at 60 s after adding BAEE. *Solid line*, *Tib*^{i5} (*G. soja* line L24); *broken line*, *Tib* (*G. max* 'Tachisuzunari')

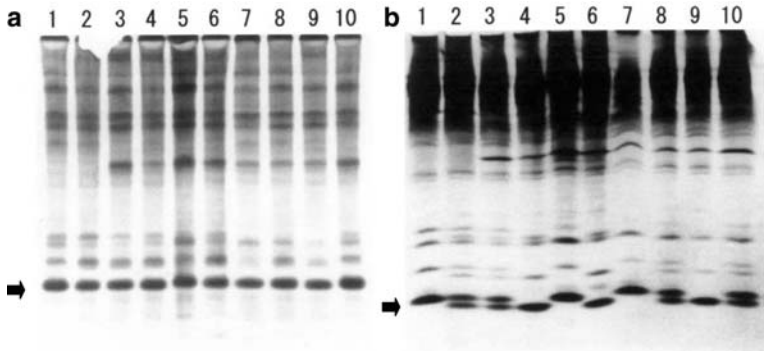

Fig. 4 Segregation patterns of the Tib^{i5} variant in F_2 seeds derived from F_1 between Tib^{i5} (*G. soja* L24) and *Tib* ('Tachisuzunari') on Davis system PAGE (**a**) and on IEF-PAGE (**b**). *Lane 1* Tib^{i5} (*G. soja* L24); *lanes 2–8*, F_2 seeds. *Arrow* shows SKTI proteins

Tib^{i5} were compared with those of *Tia* and *Tib*, six nucleotides were different between Tib^{i5} and *Tia*, and the nucleotides of these mutated positions were identical to *Tib*, i.e., two transitional mutations occurred at positions +459 (T → C) and +484 (A → G) and four transversion mutations at +329 (A → T), +366 (C → A), +502 (C → A) and +553 (C → A) (Fig. 5). They corresponded to amino acid residues at 62, 74, 105, 114, 120 and 137, respectively. Of these six mutations, five resulted in substitutions of amino acids as follows: Tyr → Phe (62), Ser → Arg (74), Met → Val (114), Leu → Ile (120) and Pro → Thr (137), and a change at position 459 caused a synonymous codon. All five of these amino acid substitutions and one synonymous codon were completely in accord with those of *Tib* type (Table 3).

Discussion

There are large differences in nine amino acid residues between *Tia* and *Tib* of SKTI. The substitutions are considered to have resulted from the accumulation of several mutations. Though seven different types of SKTI proteins have been reported thus far (Singh et al. 1969; Hymowitz 1973; Orf and Hymowitz 1979; Zhao and Wang 1992; Wang et al. 2001, 2004), no transitional intermediate forms between *Tia* and *Tib* have been found. In the present study, we first detected the intermediate type of SKTI between *Tia* and *Tib*. Our successful detection of the novel variant is due to the use of IEF-PAGE, because many researchers were not able to identify this variant by Davis system PAGE, which has usually been used to detect polymorphism of SKTI. In fact this variant shows the identical electrophoretic mobility to *Tib*.

The novel variant is confirmed to be one of the SKTI variants by genetic analysis and inhibitory activity to trypsin. The various types of SKTI are inherited as codominant alleles in a multiple allelic system at a single locus (Hymowitz and Hadley 1972; Orf and Hymowitz 1979; Wang et al. 2001). The present study revealed that the novel variant is controlled by a codominant allele to *Tib*. Though we did not examine its genetic relationships with other types, our results indicate that the novel variant type is one of the multiple alleles at the SKTI locus. This variant also showed the inhibitory activity to trypsin, whose inhibitory effect is similar to *Tib*. From

Table 1 The geographical location of 11 wild soybean (*G. soja*) accessions of japan showing a noval variant of SKTI proteins

No.	Accession	Collection site	Geographic location
1	No.336-1	Nishisenboku, Akita Pref.	140°23′E 39°32′N
2	S204	Yonezawa, Yamagata Pref.	140°06′E 37°52′N
3	S194	Yonezawa, Yamagata Pref.	140°01′E 37°49′N
4	S205	Kawanishi, Yamagata Pref.	140°00′E 37°54′N
5	No.237	Otone, Saitama Pref.	139°39′E 36°10′N
6	No.249	Iwai, Ibaraki Pref.	139°53′E 35°59′N
7	No.250	Iwai, Ibaraki Pref.	139°53′E 35°59′N
8	No.161-1	Mishima, Shizuoka Pref.	138°54′E 35°07′N
9	L24	Tsu, Mie Pref.	136°30′E 34°43′N
10	No.234	Kokubu, Kagoshima Pref.	130°48′E 31°46′N
11	No.757	Unknown	

Table 2 SKTI band segregation of F_2 seeds from the cross between *G. max* 'Tachisuzunari' (*Tib*) and *G. soja* L24 (Tib^{i5})

	No. of F_2 seeds examined	Segregation of SKTI bands			Expected	χ^2	Probability
		Tib	*Tib*/Tib^{i5}	Tib^{i5}			
G. max 'Tachisuzunari' (*Tib*)×*G. soja* L24 (Tib^{i5})	100	26	45	29	1:2:1	0.54	$0.3 < P < 0.5$

Table 3 The difference of amino acid residues among *Tia*, *Tib*, *Tic* and *Tib*^{i5} of SKTI proteins and hypothesis of evolutionary differentiation from prototype *Tia* to *Tic* and via *Tib*^{i5} to *Tib*

SKTI type	Amino acid residues									
	12	13	55	62	71	74	114	120	137	176
Tic	Glu	Asn	**Glu**	Tyr	His	Ser	Met	Leu	Pro	Leu
			↑							
Tia	Glu	Asn	Gly	Tyr	His	Ser	Met	Leu	Pro	Leu
				↓		↓	↓	↓	↓	
Tib^{i5}	Glu	Asn	Gly	**Phe**	His	**Arg**	**Val**	**Ile**	**Thr**	Leu
	↓	↓			↓					↓
Tib	**Asp**	**Ser**	Gly	**Phe**	**Asn**	**Arg**	**Val**	**Ile**	**Thr**	**Val**

these results, we propose the genetic symbol *Tib*^{i5} for this novel electrophoretic form of SKTI.

SKTI gene consists of 181 amino acids of a mature SKTI, the signal region of 25 amino acids at the N terminus and 11 amino acids at the C terminus (Song et al. 1993; Wang et al. 2001, 2004). Our results indicated that the *Tib*^{i5} gene is the same length as other types of SKTI, but has an intermediate sequence trait between *Tia* and *Tib*. Of the nine amino acid residues differing between *Tia* and *Tib*, five amino acids in *Tib*^{i5} type were different from those in *Tia*, but identical to those in *Tib*. In addition, in the codon of the amino acid at 105 residue (valine) which is synonymous between *Tia* (GTT) and *Tib* (GTC), that of *Tib*^{i5} was identical to that of *Tib*, not *Tia*. These results indicate that *Tib*^{i5} is a transitional type between *Tia* and *Tib*.

Tib^{i5} type is widely distributed throughout seven separate areas from the northeast to the southwest of Japan with a 1.5% frequency of total materials examined. These lines showed the diversification of other char-

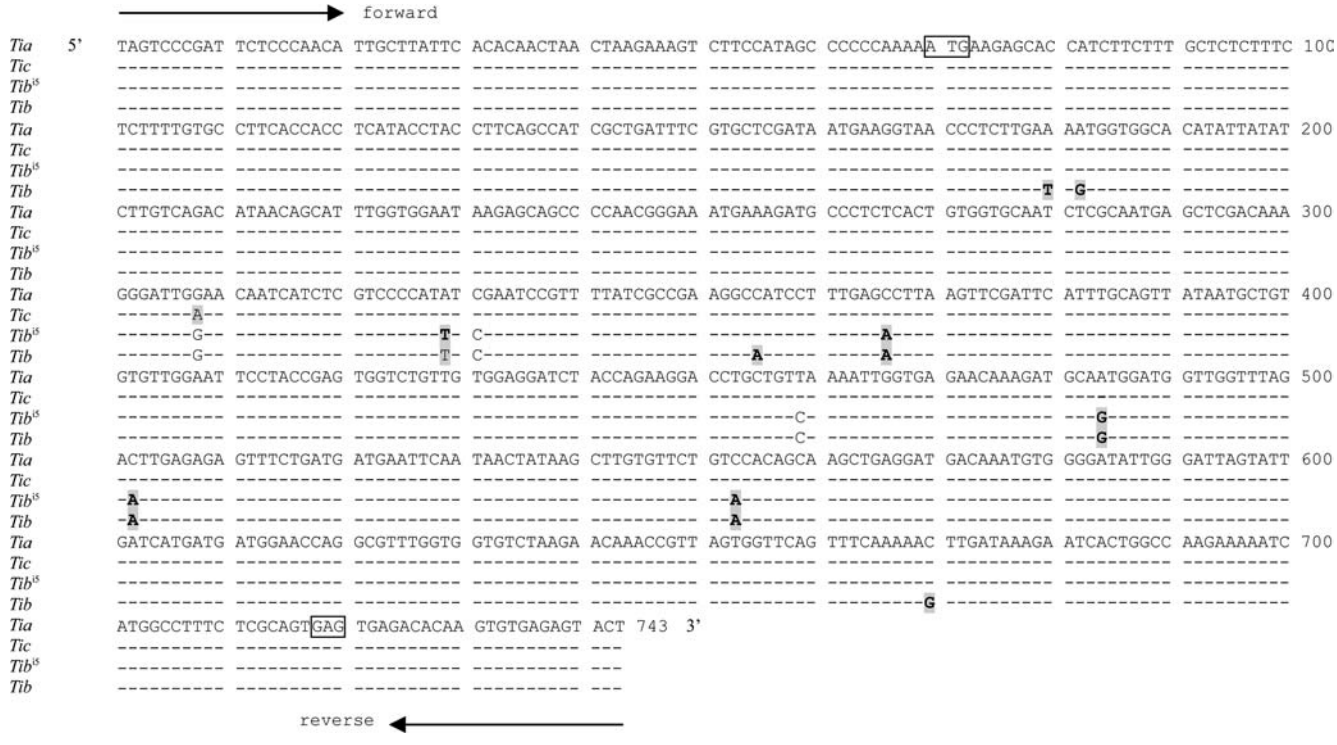

Fig. 5 Comparison of nucleotide sequences of the SKTI genes of *Tia* ('Rikuu No. 27'), *Tib* ('Tachisuzunari'), *Tic* ('Raiden') and a novel intermediate variant *Tib*^{i5} . *Letters highlighted* indicate the nucleotides that caused the changes of amino acids in comparison with *Tia*. *Boxes* indicate the start codon and the end one of the translation. *Horizontal arrows* show the positions of primers used to amplify the sequences

acteristics such as flowering time, mature period and seeds. This indicated that Tib^{i5} type did not originate from a recent mutation event, but is a universally existing independent type and spread in wild soybean from ancient times.

Three well-known SKTI types, *Tia*, *Tib* and *Tic*, are found in both cultivated and wild soybean. From these results, it is considered that the diversification of the three types of SKTI had already been completed before the speciation of cultivated soybean from wild soybean. Kaizuma et al. (1980) considered that *Tia* is the prototype from which the *Tib* and *Tic* derived, and that differentiation of *Tib* from *Tia* occurred much earlier than that of *Tic* from *Tia*. This hypothesis was supported by Kim et al. (1985). Our finding of Tib^{i5} indicates that out of nine amino acids which differed between *Tia* and *Tib*, five amino acids in *Tia* were earlier replaced resulting in Tib^{i5}, then the other four changed later resulting in *Tib*. The accumulation of mutations is considered to have proceeded gradually. Thus, we believe other transitional types, with one to eight amino acid substitutions, could also exist in wild soybean. The reason for not finding other intermediate types could be related to their rather low frequencies or unsuited identifying methods.

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