Chinese Science Bulletin 2006 Vol. 51 No. 1 54—62

DOI: 10.1007/s11434-005-0937-8

Expression and inhibitory activity analysis of a 25-kD Bowman-Birk protease inhibitor in rice

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Abstract Rice Bowman-Birk inhibitors (RBBI), with one (8 kD) or two homologous domains (16 kD), were found to be effective trypsin inhibitors *in vitro*. In this study, we demonstrate that the 25-kD protein corresponding to the three-domain RBBI indeed exists in rice *in planta*, and that the RBBIs are regulated by development and wounding. We also found by inhibitory activity assay that the 3∶13 disulfide bond, but not the 4∶5 disulfide bond, suppresses the trypsin-inhibitory activity, and the D3 domain of RBBI3-1 has no inhibitory activity against trypsin, chymotrypsin, paparin or subtilisin. Mutation analyses showed that conversion from Lys to Leu or Tyr in the N-terminal P1 site in D1 domain did not create chymotrypsin-inhibitory activity, suggesting that the structure of the reactive loop in D1 domain hinder the new inhibitory specificity at P1 site, and the chymotrypsin-inhibitory activity might need the participation of other structures, e.g. 3∶13 disulfide bond.

Keywords: Bowman-Birk inhibitor, trypsin-inhibitory activity, chymotrypsin-inhibitory activity, disulfide bond.

Bowman-Birk protease inhibitors (BBI), belonging to serine protease inhibitors, are well-studied storage and defense proteins widely existing in both dicotyledonous and monocotyledonous plants[1−3]. In dicotyledonous plants, the BBIs are about 8-kD proteins containing one domain with two reactive sites, each of which specifically inhibits trypsin or chymotrypsin[1]. Three-dimensional structures of several 8-kD dicotyledonous BBIs have been reported[4−7]. Seven disulfide bonds pull the whole protein to be a compact tertiary structure, which consists of six β-strands and the loops connecting these β-strands without any α-helix. A hypothetic axis splits the molecule into two nearly symmetric halves. Each half contains three β-strands and a reactive-site loop, which extrude the molecular and can specifically extend into the digestive pocket of trypsin or chymotrypsin[4,5,7]. In monocotyledons plants, the BBIs are either 8- or 16-kD proteins[2,8−10]. The 8-kD monocotyledonous BBIs are single-headed, with only one reactive site in the BBI domain, and inhibit only trypsin. In contrast, the 16-kD inhibitors have two homologous BBI domains with one reactive site in each domain. It is therefore thought that the 16-kD inhibitors evolved from the monocotyledonous 8-kD single-headed inhibitors[3]. Three-dimensional structure of a 16-kD barley BBI (BBBI) protein has been reported[11]. The BBBI protein folds into two compact domains similar to 8-kD dicotyledonous BBIs (designated as N and C domain, respectively), and the five disulfide bonds in each domain are a subset of the seven disulfide bonds in 8-kD dicotyledonous BBIs. The C domain of BBBI, which possesses six beta-sheets, is structurally more similar to dicotyledonous BBIs than the N domain with five beta-sheets. The largest difference between monocotyledonous and dicotyledonous BBI domains is around the C-terminal reactive-site loops corresponding to the chymotrypsin inhibitory loop of the dicotyledonous BBIs. Due to the loss of a disulfide bond near the loop as in dicotyledonous BBIs, monocotyledonous BBIs show high inherent conformational flexibility[11]. No evidence is reported that the flexible loop can bind or inhibit any type of proteases. Reactive-site loop is thought representing the most important motif in BBI protein, since such "canonical loop" structure has been found in a variety of serine protease inhibitors[12]. Some synthetic peptide mimics of BBI proteins essentially retain the three-dimensional arrangement observed in the reactive-site loop of complete native protein. This indicates that this canonical loop is an independent structural motif that does not require stabilization from the remaining part of the protein[13−15]. People even tried to screen better reactive-site-loops for the cysteine protease inhibitors by phage display[16].

* These authors contributed equally to this work.

Our previous studies found that there were at least 10 BBI genes (*RBBIs*) in the rice genome[17]. Two of the RBBIs with only one BBI domain have their P1 sites as Phe and are considered to be chymotrypsin inhibitors[18]. The other eight RBBIs (16 or 25 kD) mostly have Lys or Arg at their P1 sites, and are considered trypsin inhibitors, some of which had been confirmed *in vitro*[17,19]. However, there are still several issues to be determined for these 25-kD RBBIs. For example, the only RBBI protein sequence available (RBTI[2]) is a two-domain BBI (16 kD) that is actually the two C-terminal domains of the 25-kD member RBBI3-3[17]. This raises the first question whether the 25-kD RBBI proteins indeed exist *in planta*, or if not, whether the product is post-transcriptionally or post-translationally spliced; second, since the number of disulfide bonds is different between dicotyledous BBIs and multi-domain monocotyledonous BBIs, it would be interesting to determine whether lose of two or three disulfide bonds in the 25-kD RBBIs affects the protease-inhibitory activity; third, it would also be interesting to see whether the inhibitory specificity of RBBIs can be changed by simply replacing the amino acid residues at the P1 sites. In this study, one of 25-kD RBBI genes, *RBBI3-1*, was analyzed to answer the questions above.

1 Materials and methods

1.1 Constructs for Prokaryotic Expression

The plasmid containing *RBBI3-1* was used as the template to amplify different domains. Primers were designed as follows: D1up: 5′-CGC CGT A***GT CGA C***CA TGG GGG AAA TG-3′, D1dn, 5′-TGT ***AAG CTT*** CTA GTT CTC CGC TCG GGG-3′; D2up, 5′-GCC ***GGA TCC*** CCG ACG ACG AAG AGG-3′; D2dn, 5′-AT***A AGC TT***C ATG GCC GCG GCG TGC AC-3′, D3up, 5′-GGC ***GGA TCC*** GCA CCA CCA CGC CCG CCC-3′, D3dn, 5′-CGG ***AAG CTT*** CTA CGT CGG CTG ATC C-3′. The domain located at C-terminus of RBBI3-1 was designated as D1, the middle domain as D2 and the N-terminal domain as D3 (Fig. 1). The

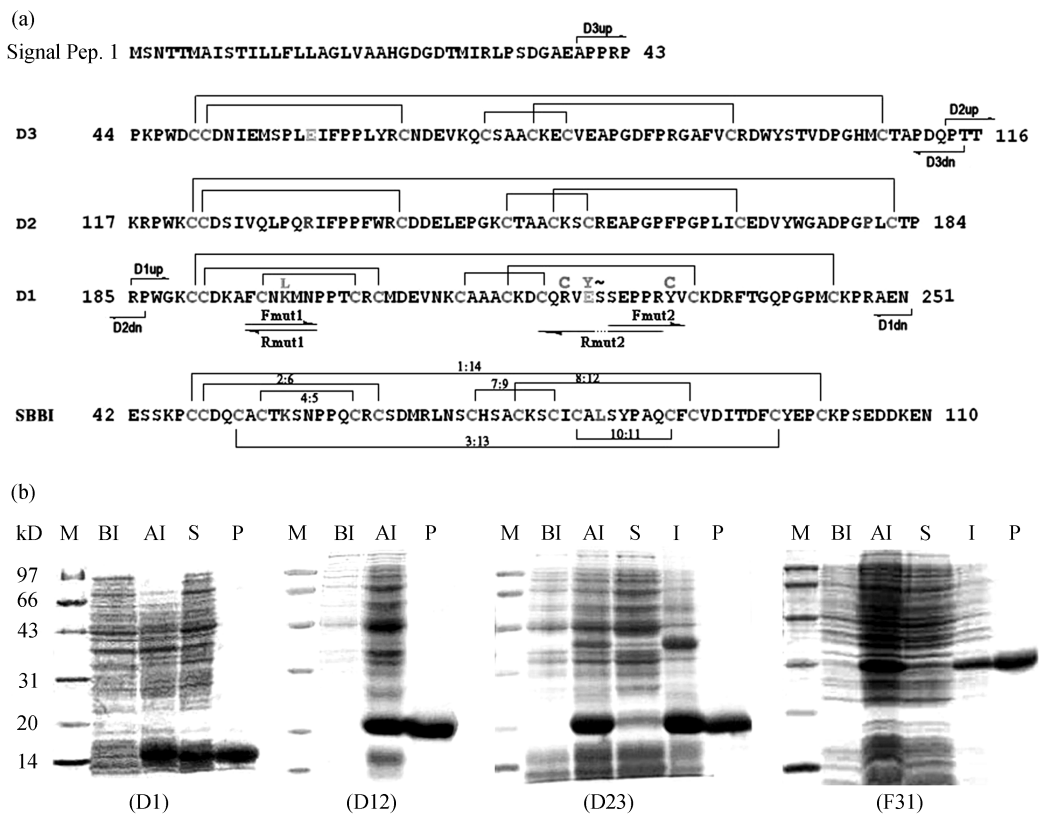

Fig. 1. The primary structures of the domains of RBBI3-1 and SBBI, and the prokaryotic expression of D1, D12, D23, F31. (a) The three repetitive domains of RBBI3-1. The positions of the primers are shown in relative to SBBI sequence. The disulfide bonds in RBBI3-1 are numbered according to the orders of disulfide bonds in SBBI. (b) Prokaryotic expression and SDS-PAGE analysis of RBBI3-1 domains. D1, domain 1; D12, domain 1 and 2; D23, domain 2 and 3; F31, domain 1, 2 and 3. M, molecular weight markers; BI, total protein of *E. coli* with plasmid before induction; AI, total protein of *E. coli* with plasmid, after induction by 0.4 mmol/L of IPTG; S, soluble proteins; I, inclusion bodies solubilized by 8 mol/L urea; P, about 5 μg of purified and renatured protein product.

Table 1 List of different constructs

Clones	Vectors	Inserted DNA Fragment			Predicted protein size (kD)
		primers	domains	size (bp)	
pET28-D1	pET28a (*Sal* I + *Hin*d III)	D1up (5′), D1dn (3′)	D1	200	11.8
pET28-D12	pET28a (*Bam*H I + *Hin*d III)	D2up (5′), D1dn (3′)	D1+D2	417	19.0
pET28-D23	pET28a (*Bam*H I + *Hin*d III)	D3up (5′), D2dn (3′)	D2+D3	446	19.9
pET28-F31	pET28a (*Bam*H I + *Hin*d III)	D3up (5′), D1dn (3′)	D1+D2+D3	642	27.4

DNA fragments were amplified and cloned into pET28a as described[17]. The target fragments to be amplified and the recombinant plasmids were shown in Table 1.

For mutations, two pairs of mutated primers were designed as follows: Fmut1: 5′-C TTC TGC AAC CTG ATG AAC C-3′, Rmut1: 5′-G GTT CAT CAG GTT GCA GAA G-3′; Fmut2: 5′-TCG GAG CCG CCT CGC TGC GTC-3′, Rmut2: 5′-GCG AGG CGG CTC CGA ATA CAC ACA CTG GCA-3′, in which changed bases were underlined. In the same time, two primers T7p (5′-TTA ATA CGA CTC ACT ATA GG-3′) and T7t (5′-CTA GTT ATT GCT CAG CGG-3′), were designed in the T7 promoter and terminator regions of pET28-D1 respectively. The mutated primer pairs (Fmut1 and Rmut1) will change the P1 site of D1 domain from K (198) to L (Fig. 1), the construct of which was designated pET28-D1M1. Fmut2 and Rmut2 will change the 222－231 sequence in D1 domain from RVESSEPPRY to CVY-SEPPRC (The dash represents deletion of one residue, Fig. 1), the construct of which was designated pET28-D1M2. All the recombinant plasmids were verified by sequencing.

1.2 The expression, refolding and purification of the fusion proteins

The expression of these proteins in *E. coli* was carried out as described[17,19]. The proteins F31, D12, D23 and D1M2 were not soluble in this study possibly due to the multiple disulfide bonds, and therefore inclusion bodies were harvested. Protein refolding and purification were carried out as previously described[19]. As for the soluble D1 and D1M1 fusion proteins, the cells were collected, lysed and mixed with 5 mL of Ni^+-charged His-Bind resin for 30 min. After washing, the resin was finally suspended in buffer A (20 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 8.0) packed into a column, and further washed. The fusion proteins were eluted with imidazole according to manufacturer's instruction (Novagen).

1.3 Western blot

F31 fusion protein was used to immunize Rabbits and serum was extracted as the primary antibody for western blot hybridization. About 0.2 gram of rice seeds (*Oryza sativa* L. ssp. *indica* var. Zhonghua11) germinated for 48 h was grounded at 4℃ and extrated with extraction buffer (50 mmol/L phosphate, 10 mmol/L 2-ME, 0.1% Trion X-100, 1 mmol/L EDTA, pH=7.5) before centrifugation (12000 rpm, 5 min). The concentration of the proteins was determined as described [20]. About 30 μg of rice protein and 100 ng of purified F31 and D12 were loaded in SDS-PAGE analysis. Western blot was performed with BCIP and NBT (Promega) second antibody according to manufacturer's instruction (Promega).

1.4 Inhibitory Activity Assay of fusion proteins

Inhibitory activities against trypsin 1∶250 (Difco Laboratories, Detroit) and chymotrypsin (C4129, Sigma-Aldrich, St. Louis) were determined by incubating the purified fusion protein with the enzymes at 25℃ for 5 min. The remaining trypsin activity was measured with N-tosyl-L-arginine methyl ester (T4626, TAME, Sigma-Aldrich) as the substrate as described[21]. The remaining chymotrypsin activity was measured with N-acetyl-L-tyrosine ethyl ester (A6751, ATEE, Sigma-Aldrich) as the substrate as described before[22]. The absorbency at 247 nm (for ATME) and 237 nm (for ATEE) was determined on a GBC Cintra 10e UV-Visible spectrometer (GBC, Melbourne). The commercial soybean Bowman–Birk inhibitor (T9777, Sigma-Aldrich) was assayed as the positive control.

1.5 Determination of kinetic constants

K_m value for the hydrolysis of TAME by trypsin was determined as described[23,24]. Three paralleled experiments were performed for each plot. Initial reaction speed was determined by the absorbance curve from 0 to 15 s. Both Lineweaver-Burk plots and Eadic-Hofstee plots methods were adopted to calculate kinetic con-

stants. The linear fit and other calculation was carried out by using Origin6.1 software.

The effects of inhibitors on trypsin were determined as described above with some modifications (33 nmol/L of trypsin and 10—80 nmol/L of inhibitors in 1 mL of the spectrophotometer assay). Kinetic constant (K_i, apparent K_m) was calculated by Lineweaver-Burk kinetic analysis. After a K_i value was determined under a certain inhibitor concentration, three or four different concentrations were applied independently to confirm the result.

2 Results

2.1 RBBI proteins with one, two and three domains are developmentally regulated

High level expression in *E. coli* was obtained for the fusion proteins D1, D12, D23, F31, D1M1 and D1M2 (Fig. 1(b), expression of D1M1 and D1M2 were similar to D1, not shown in Fig. 1(b)). Western blot showed that four protein bands of different sizes, 25, 19.5, 18.5 and 9 kD, were detected by F31 antibody in rice seeds during early germination (Fig. 2(a)). Since one amino acid residue is about 110 Da, the 25-kD protein is predicted to contain about 230 amino acid residues, larger than the 195 residues of RBTI[2,25]. In the same time, the three-domain RBBIs predicted from DNA sequence, i.e. RBBI3s, are about 260 amino acid residues in average[17]. Taking into consideration that a signal peptide of about 30 amino acid residues will be cut off in the process of protein maturation, the protein corresponding to the 25-kD band represents the mature three-domain protein which is about 230 amino acid residues in size. This result is the first direct protein evidence that there are native three-domain RBBI proteins in rice seeds. The 19.5-kD and 18.5-kD protein bands corresponded to those mature two-domain RBBI2s whereas the 9-kD band represented those mature single-domain RBBIs (Fig. 2(a)).

The RBBI proteins are developmentally regulated (Fig. 2(b)). The level of RBBIs, both two-domain and three-domain members, are high during early stage of germination, and declined dramatically four days after imbibition, whereas the level of one-domain is low (Fig. 2(b)). In rice seedlings and mature plants, the three-domain RBBI proteins are detectable, although low. However, the two-domain and one-domain RBBIs are not detectable (Fig. 2(b)). This result suggests that the RBBIs are mainly required for early germination.

To clarify the role of RBBIs in the defense response, we examined the protein level of RBBIs in rice leaves during the wounding treatment. It was clearly shown that the three-domain RBBIs were induced 12 h after the treatment and remained at a stable expression

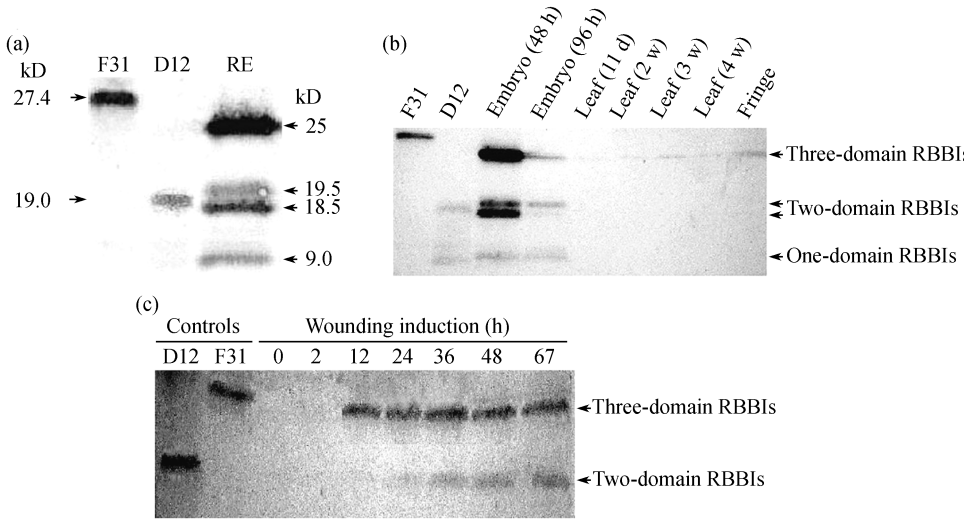

Fig. 2. Western analysis on RBBIs. (a) RBBIs in rice germinating seeds by Western blot analysis *in vivo*. About 30 μg of proteins isolated from rice germinating seeds was loaded. 100 ng of F31 and D12 fusion protein were loaded as positive controls and protein molecular weight markers. RE, rice germinating seeds. (b) RBBIs during rice development by western blot analysis. About 30 μg of proteins per lane was loaded. 100 ng of F31 and D12 fusion protein were loaded as positive controls and protein molecular weight markers. RE (48 h) and (96 h), rice germinating seeds 48 hours and 96 hours after imbibition respectively; Leaf (11 d), (2 w), (3 w) and (4 w), leaves from rice plants of 11 d, 2 weeks, 3 weeks and 4 weeks, respectively. (c) Induction of RBBIs by wounding. Leaves from 4-week rice seedlings were cut and put into 100 μmol/L of jasmonic acid (JA) in dark before they were collected at different intervals.

level (Fig. 2(c)). Two-domain RBBIs are gradually induced by wounding as well, but 12 h later than that in three-domain RBBIs (Fig. 2(c)). No signal was detected for the one-domain RBBIs (data not shown).

2.2 All the fusion proteins possess inhibitory activity against trypsin but not chymotrypsin

In order to determine the inhibitory activity and specificity of D1, D12, D23 and F31, the titration experiments were conducted. As shown in Fig. 3, all the four proteins showed inhibitory activity against trypsin but not to chymotrypsin. When equal molar of the inhibitors were added, the trypsin-inhibitory activities are F31≈D12 > D1 > SBBI > D23. Since the D3 domain has Glu in its P1 site and has no trypsin-inhibitory activity, the trypsin-inhibitory activity of D23 is most likely contributed by D2. It is worth noting that although D2 lost its disulfide bond at the bottom of the reactive loop, D2 possessed trypsin-inhibitory activity (Fig. 3). Moreover, when concentration of the D1 is higher than a certain value, its trypsin-inhibitory activity is higher than that of SBBI in equal molar (Fig. 3). The results from above indicate that the disulfide bond 10∶11 in the C-terminal domain (Fig. 1) hardly affects the trypsin-inhibitory activity of N-terminal domain, and that the absence of the disulfide bond 3∶13 can increase the trypsin-inhibitory activity. The absence of the disulfide bond 4∶5 results in a huge loss of trypsin-inhibitory activity, but this could be complemented partly by the proline-formed turn structure.

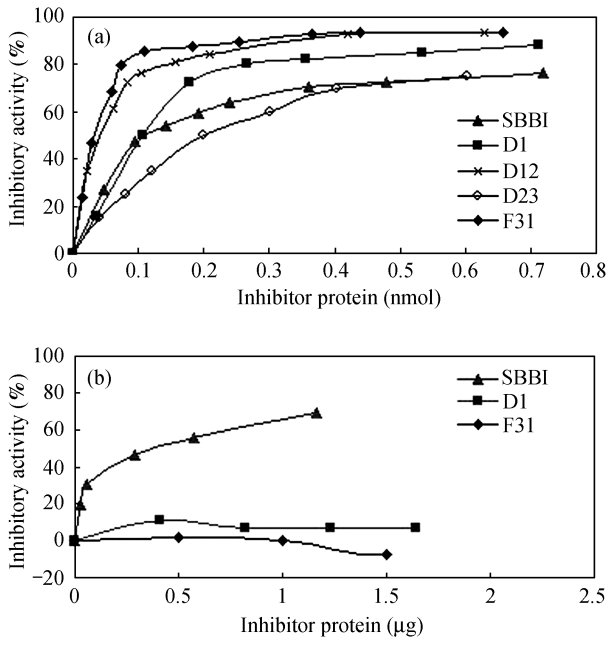

Fig. 3. Titration of trypsin and chymotrypsin with various amounts of fusion proteins and soybean Bowman-Birk inhibitor (SBBI). (a) A fixed amount of trypsin (100 TAME units, i.e. ΔA_{247}/min = 0.10) was incubated with increasing amounts of inhibitors in 0.2 mL of Tris-HCl buffer (50 mmol/L, pH = 8.0) for 10 min at 25℃. Reduction of trypsin activity was then determined. The reaction volume was 1 mL. (b) A fixed amount of chymotrypsin (2.0 μg) was incubated with increasing amounts of inhibitors in 0.2 mL of Tris–HCl buffer (50 mmol/L, pH = 7.5) for 10 min at 25℃. Reduction of chymotrypsin activity was then determined. The reaction volume was 1 mL.

However, no chymotrypsin-inhibitory activity was detected for the two D1 mutants of (data not shown). This suggests that there could be other factors involved in inhibitory specificity.

2.3 The concentration of inhibitors will affect inhibitory constants

We adopted the Lineweaver-Burk plots and Eadic-Hofstee plots to calculate K_m, the affinity constant between enzyme and its substrate. As a pilot experiment, we used these two methods to calculate the K_m of trypsin with TAME. The result is K_m = 8.75 μmol/L, which is consistent with previously reported values for trypsin-catalyzed hydrolysis of TAME[23,24], indicating the reliability of the system we used.

We found that the Lineweaver-Burk plots for inhibitors D1, D12, D23, F31 and the control SBBI showed a typical pattern of competitive inhibition (Fig. 4(a)—(e)). According to the mechanism of competitive inhibition, following equations were used to calculate the inhibitory constant:

$$\begin{array}{l} \mathrm{E} \underset{}{\overset{}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{\text{Competitive}} \mathrm{E+P} \\ K_i \Big\Updownarrow \quad K_i = [\mathrm{E}][\mathrm{I}]/[\mathrm{EI}] \\ \mathrm{EI} \end{array} \qquad v = \frac{V_s}{K_m(1+i/K_i)+s} = \frac{V_s}{K_m^{app}+s}$$

$$K_m^{app} = K_m(1+i/K_i)$$

Note: changes K_m, no effect on V

Competitive inhibitors decrease the selectivity of enzyme (i.e. apparent V_{max}/K_m) by factor of $(1 + [I]/K_i)$. With the initial K_m discussed above, inhibitory constants (K_i) of D1, D12, D23 and F31 were calculated (Table 2). Among the inhibitors, F31 shows the highest binding affinity to the enzyme (K_i = 4.2 nmol/L), suggesting that F31 possesses the strongest inhibitory activity. D23 (K_i = 8.1) showed the lowest inhibitory activity while D1 (K_i = 7.01) and D12 (K_i = 5.74) stayed in the middle. When single BBI domains are compared, the binding capacity to trypsin is SBBI > D1 > D2 (D3 has no trypsin-inhibitory activity), which differs from the result obtained from the condition with inhibitors of higher concentration (Fig. 3).

Table 2 K_i value of fusion proteins and SBBI

	F31	D12	D23	D1	SBBI
K_i (nmol/L)	4.24±0.02	5.74±0.94	8.08±0.52	7.01±0.12	6.0±1.2

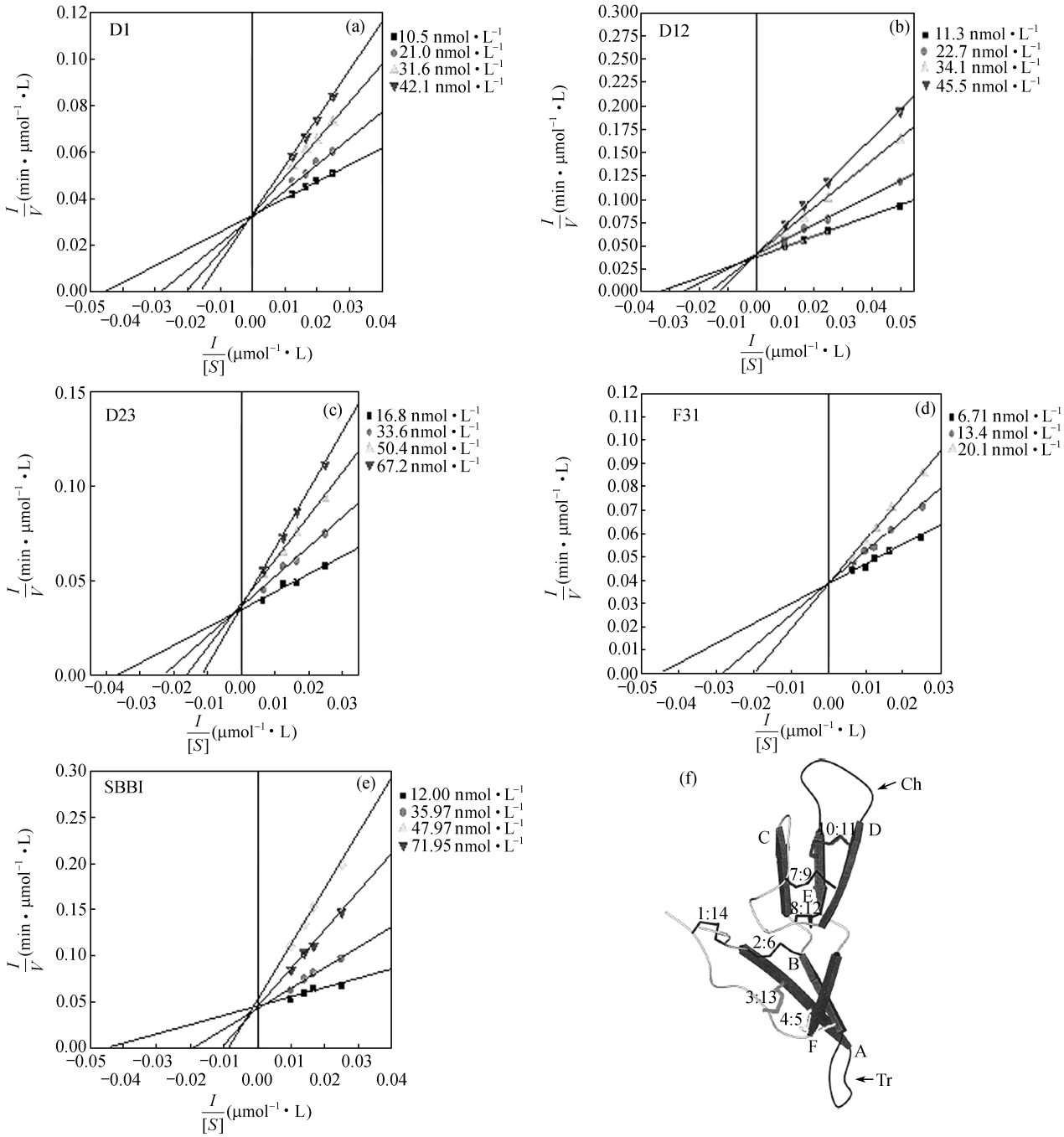

Fig. 4. Lineweaver-Burk curves of RBBIs and SBBI, and 3D structure of SBBI. (a)—(e) Lineweaver-Burk plots for inhibitors D1, D12, D23, F31 and SBBI respectively. (f) Three dimensional structure of SBBI (based on ref. [28]). The numbering of disulphide bonds is based on the order of cysteines in SBBI. Tr stands for the trypsin inhibitory site in the N-terminal domain, while Ch stands for chymotrypsin-inhibitory site in the C-terminal domain.

3 Discussion

This study has provided information to the answers to the three questions raised in the introduction.

For the first time, by western blot, we proved that the 25-kD RBBIs, a member of which was once considered as 16-kD RBBI[2,25,26], existed naturally in rice, and that this protein may represent a three-domain RBBI. Furthermore, the RBBIs are developmentally regulated and induced by wounding, suggesting that RBBIs are important for the rice seed germination and are required for the defense system.

When compared the trypsin-inhibitory activity of D1 with that of SBBI, it is found that, although the K_i of SBBI to trypsin is the lowest, the inhibitory activity of D1 is higher than that of SBBI when the inhibition ratio is over 50% (Fig. 3). This suggests that the binding of D1 to trypsin at higher concentration does not meet the dissociation constant at lower concentration and that the disulfide bond 3∶13 in BBIs is not necessary for the trypsin-inhibitory activity. D1 domain of RBBI3-1 is similar to the barley two-domain BBI, lacking the two disulfide bonds 3∶13 and 10∶11 in SBBI (Fig. 1). The disulfide bond 10∶11 is at the base of the C-terminal loop (Fig. 4(f), the blue-colored disulfide bond), and is considered to maintain the rigid structure of the loop and barely affects the reaction site in N-terminus. Meanwhile, the 3∶13 disulfide bond is considered to fix the β-sheet A with C-terminus of SBBI (Fig. 4(f), the red-colored disulfide bond). If this disulfide bond is lost, the β-sheet F would be in looser constraint and could cooperate with β-sheet A and B to form a conformation much more helpful for trypsin-inhibitory activity. However, interestingly, the 3∶13 disulfide bond could be found in all the dicotyledonous BBIs (Fig. 5), suggesting that the existence of 3∶13 disulfide bond may be necessary for maintaining other function of the dicotyledonous BBIs.

In the titration assay with trypsin, we found the titration curve of D12 almost overlap with that of F31 (Fig. 3), suggesting that, as predicted, D3 domain of RBBI3-1 has no trypsin-inhibitory activity since the P1 residue in D3 domain is Glu that has no reported protease-inhibitory activity. Therefore, the trypsin-inhibitory activity exhibited in D23 is most likely from the D2 domain. Comparing with D1 domain, D2 domain lost its disulfide bond 4∶5 (Fig. 1(a)). The fact that D1 has stronger trypsin-inhibitory activity than D2 suggests that the disulfide bond 4∶5 contributes to improving the trypsin-inhibitory activity, but it is not necessary for activity maintenance. It was found that the residues in most P1 sites of D2/D3 domains are Pro and those in the corresponding sites of D1 domains and dicotyledonous BBIs are Cys (Fig. 5). It is possible the Pro in the P1 site of D2/D3 domain that may maintain necessary turn corner structure and therefore maintain the trypsin-inhibitory activity.

Our study also showed that changing the P1 site of BBI is not an efficient way to change the specificity of the inhibitors. In mutant D1M1, the P1 site was changed from Lys to Leu (since SBBI has Leu in the P1 site determining the chymotrypsin-inhibitory activity), but no chymotrypsin-inhibitory activity was detected. The sequence alignment analysis shows that the amino acid residues at the N-terminal reactive P1 sites are conserved, either Arg or Lys, among all the dicotyledonous BBIs and the monocotyledon BBI domain 1 (D1s) (Fig. 5), with the only exception of PVUBBI2 with an Ala at the P1 site. This is not in agreement with the hypothesis that the amino acid residues at the P1 sites of the trypsin inhibitors are highly variable[27]. In contrast, the P1 sites are much more flexible in D2/D3 domains, which lack the 4∶5 disulphide bond, and even 4 (out of 12) P1 sites are non-alkaline amino acid residues (Fig. 5). It is likely that the 4∶5 disulphide bond may be responsible for preventing the P1 site of BBI domains from frequent changes.

In mutant D1M2, simulation of the SBBI C-terminal reaction loop in RBBI D1 does not result in chymotrypsin-inhibitor activity. We tried to reconstruct the C-terminal subdomain in mutant D1M2 by inducing the missing disulfide bond 10∶11 (Fig. 4(f), the blue-colored disulfide bond) to D1 domain and changing the Glu at P1 site into hydrophobic amino acid Tyr. These changes made D1M2 much similar to SBBI in the C-terminal activity loop in terms of amino acid sequence. However, chymotrypsin-inhibitory activity was not restored. There are several possible reasons for the result: (i) The protein with an addition disulfide bond cannot be folded correctly in *E. coli*. (ii) There could be some other factors affecting the activity restoration in C-terminal site, for example other residues on the reaction loop or the other missing disulfide bond 3∶13 in SBBI. The disulfide bond 3∶13 was proved to be not helpful for trypsin-inhibitory activity, therefore the only explanation for the conservation of this disulphide bond

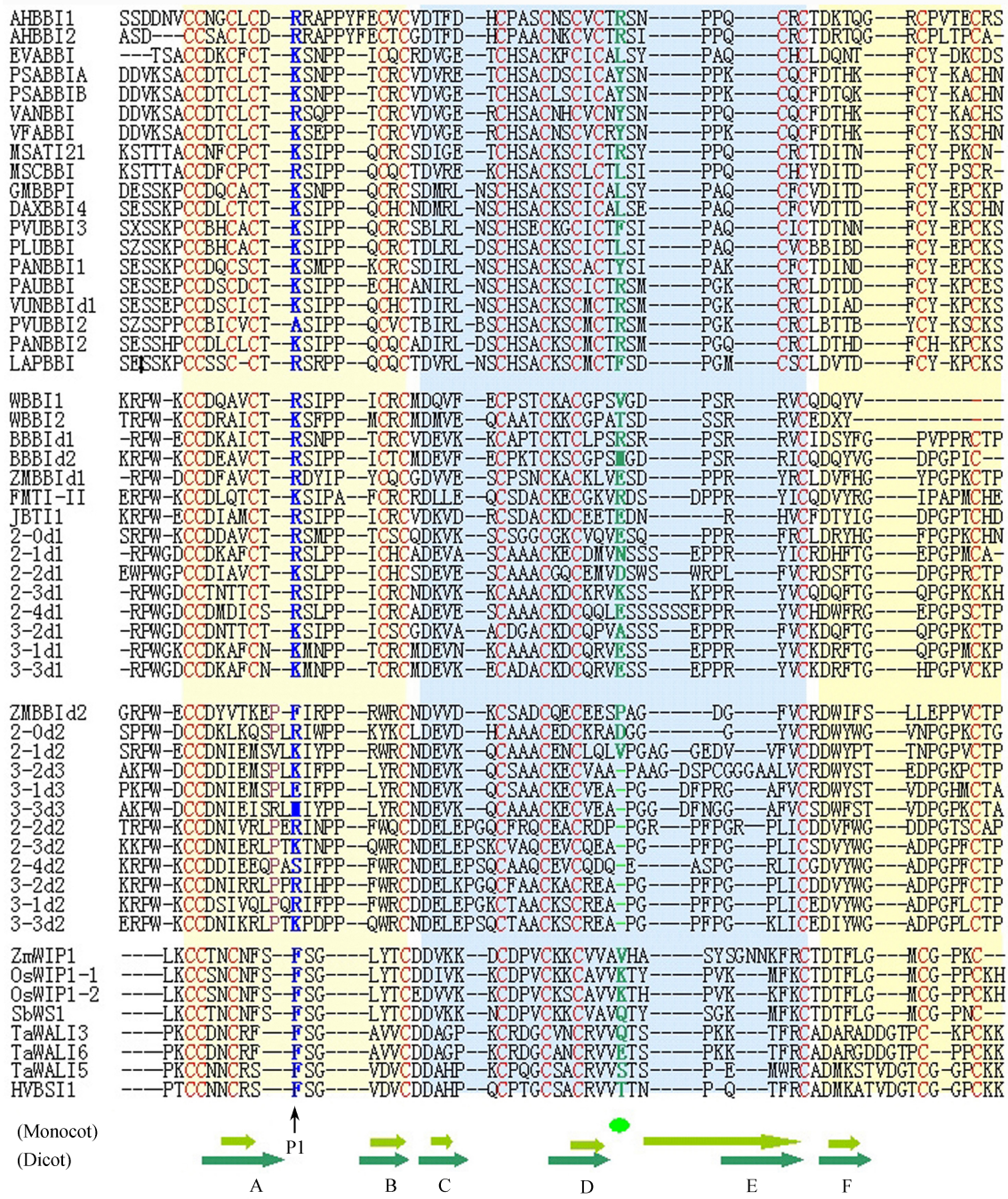

Fig. 5. Amino acid sequence alignment of plant BBIs. The plant species are listed in Supplemental Table 1. According to ref. [11], each domain can be divided into two sub-domains which are distinguished in different background colours. The arrows at the bottom represent the positions of those potential β-sheets. P1 indicates the reactive site of the domain. The green oval indicates the position of another potential reactive site.

among dicotyledonous BBIs is that this disulfide bond is necessary for maintaining the activity of the C-terminus. More mutations have to be made for other amino acid residues and for other disulfide bonds to further clarify the mechanism behind the trypsin- and chymotrypsin-inhibitory activity.

Acknowledgments The authors are grateful to Prof. Jin

Changwen and Xia Bin (NMR Center, Peking University) for technical help. This work was supported by the State Key Basic Research and Development Plan (Grant Nos. G2000016200 & G1999011602), the National High-Tech Program of China (Grant No. 2002AA2Z1001-07), and by the Rockefeller Foundation (Grant No. 97003).

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(Received June 13, 2005; accepted September 19, 2005)