Studies on transforming *Brassica napus* L. with proteinase inhibitor gene *DsTI1* from *Descurainia sophia*

DU Wenmimng, HUANG Ji, ZHANG Hongsheng, GUAN Rongzhan, DONG Haibin, TANG Sanyuan

National Key Lab of crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, P. R. China.
Email: guanrzh@njau.edu.cn

Abstracts

By sequencing large amounts of cDNA from *Descurainia sophia* silique cDNA library, we got a full-length proteinase inhibitor gene *DsTI1* encoding a protein of 102 anmino acids containing a conserved functional reactive site"CAPRIFPSFC". The similarity of DsTI1 to ATTI2 (*Arabidopsis thaliana*), RTI (*Brassica napus*), *MTI-2*(*Sinapsis alba*) was 78%, 61% and 61%, respectively. By *Agrobacterium tumefaciens* -mediated transformation, *DsTI1* was integrated into rapeseed variety NJ5424. We obtained transgenic individuals, and its transformation efficiency was 1.4%. Expression of the gene in transgenic plants was confirmed by RT-PCR. Bioassay of insect resistance to the artogenia (*Pieris rapae* L.) larvae revealed that transgenic plants had significantly increased resistance.

Key words: DsTI1 gene, Brassica napus Insect resistance, *Descurainia sophia*

Proteinase inhibitors (PIs) have been found in seeds and storage organs of a wide range of plants, playing an important role as defense proteins in plants defense mechanism against insect and pathogen attacks [1]. According to the type of inhibited proteolytic enzyme, PIs are classified into four classes: serine-, cysteine-, aspartic-, and metallo-PIs. Several serine PIs have been indicated to inhibit the growth of larvae from lepidopteran insect [2]. Serine PIs (or TI) are the major proteinase present in plants, and used for processes such as protein mobilization in storage tissues. Serine PIs contain a cysteine residue as the catalytically active nucleophile in the enzyme active site, showing specificity to trypsin and/or chymotrypsin. Serine PIs in general are small, stable, abundant proteins, particularly abundant in storage tissues such as seeds, tubers, leaves, endosperm and fruits [3]. These inhibitors also functioned as endogenous regulators of proteolytic activity and an important factor in response to abiotic or biotic stress [4]. By inhibiting activity of digestive proteinase, serine PIs can damage the epithelial lining of the mid-gut, and retard the development of insect pest which ingested in plant leaf. In transgenic plants, it was observed that overexpressing serine PIs is more resistant to insect than control plants [5].

Cruciferous serine PIs represent a novel serine PI family, and are different from serine proteinase inhibitor structure reported in other plant family. Cruciferous proteinase inhibitor genes *MTI-1* and *MTI-2* were firstly isolated from *sinapsis alba*. PI gene *RTI* from *B. napus,* encodes a protein of 60 residues, and has similar functions to *MTI-2* [6]. In *A. thaliana*, seven proteinase inhibitors have been identified. *RTI*, *MTI-2* was demonstrated to be insect-resistant genes, respectively.

We firstly constructed the cDNA library of *D. Sophia* silique, then isolated cDNA of proteinase inhibitor designated as *DsTI1* from the library, and transformed rapeseed with this gene to evaluate its function.

1 Materials and methods

1.1 Plant materials and reagents

Siliques of *D. sophia* plants grown in the experimental fields of Nanjing Agricultural University, were harvested 15 days before maturation, frozen in liquid nitrogen, and stored immediately at -80°C for subsequent processing.

1.2 cDNA Library Construction

Total RNA was extracted, subsequently separated and purified using Oligotex mRNA Mini Kit. *D. sophia* silique cDNA library was constructed by using SMART^{TM} technology (Clontech). One hundred positive plaques were chosen randomly and sequenced after converting the λ TriplEx2 clone to TriplEx2 plasmid. Determination of the nucleotide sequence of the cDNA clone was performed using universal primers T7. The sequences were used for Blast search in the database at NCBI (http://www.ncbi,nlm.nih.gov) by using the advanced BLAST program.

1.3 Sequence analysis of DsTI1 cDNA

DsTI1 cDNA sequences were analyzed by DNAssist 2.0, the multiple protein sequences alignment and phylogenetic tree were generated by Clustal W and Clustal X, respectively.

1.4 Vector construction

Plant expression vector was constructed as pCAMBIA1301-*DsTI1* containing *DsTI1* gene and hygromycin phosphotransferase (*Hpt*) gene promoted by CAMV 35S. Hygromycin was used for transgenic selection. All manipulations

were performed with the standard procedures [7].

1.5 Transformation of rapeseed

A. tumefaciens strain LBA 4404 was streaked on YEB solid medium with 50mg/L kanamycin and 50mg/L rifampicin in order to obtain single colonies. Bacterial cultures of single colonies were grown overnight at 28°C and shaken at 180rpm in YEB liquid medium supplemented with 50mg/L kanamycin. The suspension of bacteria was then diluted in 1:40(v/v) with fresh YEB medium at the same cultivation until OD_{600nm}=0.5. The bacteria were collected and washed twice, then were resuspended at an OD_{600nm} of 0.3--0.4.

Rapeseed seeds of variety NJ5424 with surface sterilization were placed on modified MS medium (MS medium supplemented with IBA 4mg/L, 3% sucrose and 0.7% agar) in petri dishes, which kept in the dark for 2 days until the onset of germination and maintained in a greenhouse at 25°C, with a 16:8h light/dark cycle. Cotyledonal petioles were excised approximately 3mm segments from 5 day-old sterile seedlings and infected by OD_{600nm} of 0.3--0.4 *Agrobacterium* for 10min. After co-cultivation, the explants were rinsed with sterile distilled water containing 500mg/L carbenicilin, and transferred to the selective regeneration medium (modified MS medium supplemented with 12.5mg/L Hygromycin, 500mg/L Carbencillin, 3mg/L 6-BA, 0.1mg/L NAA). All explants were subcultured every 10-15days on selective regeneration medium until visible shoots formed. The regenerating shoots were excised from the primary explants, elongated on selective regeneration medium with Hygromycin and Carbencillin. Subsequently, they were incubated in rooting medium (modified MS medium with 0.1mg/L NAA, 0.1mg/L 6-BA, 12.5mg/L Hygromycin, 200mg/L Carbencillin). These plantlets were considered to be hygromycin-resistant and were transferred to the greenhouse at 25°C, with a 16:8h light/dark cycle. Transformation frequency was defined as percentage of infected explants that produced hygromycin-resistant plantlets.

1.6 PCR analysis

Genomic DNA was extracted from leaves of untransformed plants and putative transgenic plants [8], and PCR were carried out. Primers used for amplification of the *DsTI1* were used as following: 5′-GATGGCTATGACAATGAAGT-3′and 5′-ACATAAACCATGACACATGC-3′for a 323bp portion of *DsTI1* gene. The amplification protocol for *DsTI1* was 94°C for 5min followed by 30cycles of 94°C for 1min, 54°C for 1min, and 72°C for 1min.Cyclings were followed with an incubation of 72°C for 10min. The specific oligonucleotide primers 5′- ATGAAATCACGCCATGTAGT -3′ and 5′-ACTATCCTTCGCAAGACCTT -3′ were used to amplify *Hpt* gene. The amplification program was used as following: denaturation at 94°Cfor 5min firstly, followed denaturation for 40s at 94°C, annealing for 40s at 54°C and extension for 1min at 72°C, 35 cycles, extending for 10min at 72°C. The amplified fragments were electrophoresed on a 1% agarose gel and detected by ethidium bromide staining.

The expression of *DsTI1* gene was detected by RT-PCR analyses with primer pair same as described above. Amplification products were electrophoresed on a 1% agarose gel, and then visualized by ethidium bromide staining.

1.7 Insect bioassays

A laboratorial population of *Pieris rapae* L. was reared in growth chamber for several generations. Seedlings were kept in a 16h/8h light/dark photoperiod, at 25°C. Control plants were selected, which were similar to the transformants used in experiment. The trays were fitted with a moistened filter paper to keep the leaf fragments humid. Five neonate artogenia larvae were placed in each tray; the leaves were replaced every two days. The larvae were transferred from old leaves to new. Survivors were counted, and larvae were weighed at 24h intervals. Each experiment was repeated for three times.

2 Results

2.1 Full-length DsTI1 cDNA cloning

Screening *D. sophia* silique cDNA library, we got a full-length cDNA sequence highly homogous to *Arabidopsis* proteinase inhibitor (*ATTI2*), designated as *DsTI1* with 512bps long, containing a 309bp open reading frame (ORF). The deduced protein encoded a 102 amino acids (aa) with a predicted molecular mass of 11KD and an isoelectric point of 6.7.

DsTI1 cDNA encoded a polypeptide with an N-terminal 33-amino acids extension enriched in hydrophobic residues, where an N-terminal signal peptide mediates the proteinase inhibitor secreting into the lumen of the endoplasmic reticulum. The mature protein contained 8 cysteine residues which forms 4 disulfide bridges , and agreed with the conclusion of MTI、RTI、ATTI [6, 9, 10]. The founction of the 8 residues at the C-terminus is not clear. The protein contained a sequence encoding ten-peptide “CAPRIFPT(S)FC”, which was conversed in PIs as a reactive site loop. Sequence alignment revealed that, DsTI1 presented different similarity with cruciferous PIs reported in Genbank (http://www.ncbi.nlm.nih.gov) (Fig. 1A). The similarity of DsTI1 to ATTI2 (*A. thaliana*), RTI (*B. napus*), MTI-2 (*S. alba*) was 78%, 61% and 61% identity, respectively. The DsTI1’s similarity to ATTI1 and ATTI3~7 were less than 60%. Neighbour-joining phylogentic tree was constructed (Fig. 1B).

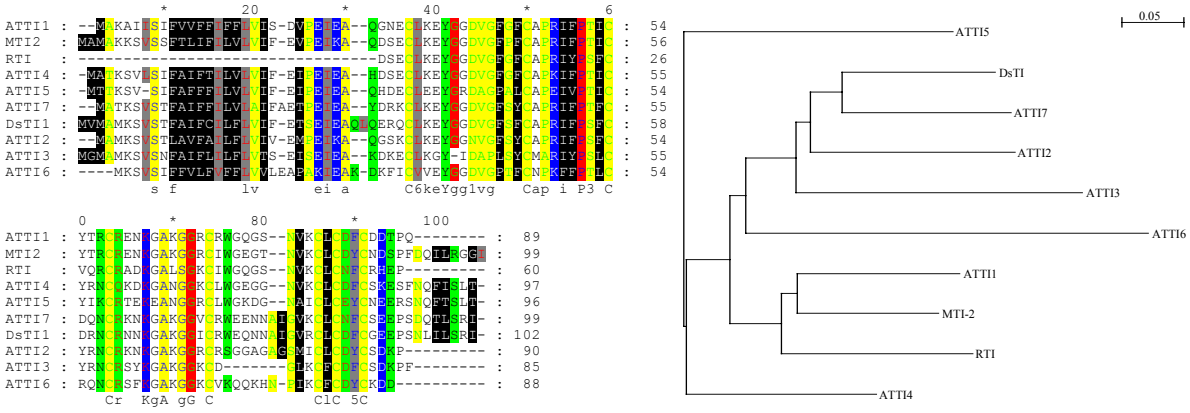

Fig. 1 Aligment of DsTI1 amino acid sequence with related cruciferae trypsin inhibitor genes

Fig. 1A. MTI-2 (CAA58994), RTI (P80301), ATTI1, ATTI2, ATTI3, ATTI4, ATTI5, ATTI6 and ATTI7 (CAG15208, CAG15239, CAG15318, CAG15190, CAG15243, CAG15242, NP-973985, respectively).

Fig. 1B. Phylogenetic tree of related cruciferae trypsin inhibitors.

2.2 Transformation process

According to rapeseed transgenic procedures mentioned above, explants from rapeseed cotyledonal petioles after infected by *Agrobacterium*, were cultured and selected for 6-8weeks. Then, regenerating shoots were obtained and subcultureed on rooting medium. Finally, seedlings were carefully transferred to aseptic soil (Fig. 2). Through this process by using 1911 explants, we got plantlets of 128.

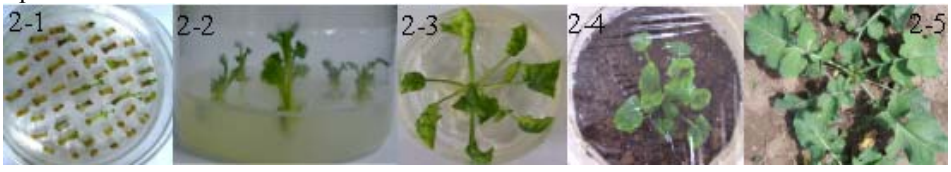

Fig. 2 Regeneration of transgenic rapesaeeds

2.3 PCR detection

To demonstrate the transformted plants , PCR analysis was carried out firstly for a rapid identification. Using primers of *Hpt* for detection of hygromycin-resistant plants, PCR amplification of the genomic DNA gave 583bp products of amplification (Fig. 3a). Further, using primers of *DsTI1* for detection of transformated plants, PCR amplification of the genomic DNA gave 323bp amplification products, which were similar to that of the positive control (Fig. 3b). Under similar conditions, 583bp band for *Hpt* and 323bp band for *DsTI1*, can not be observed in the negative control. Thus, we've demonstrated that transformation technique effected in this experiment.

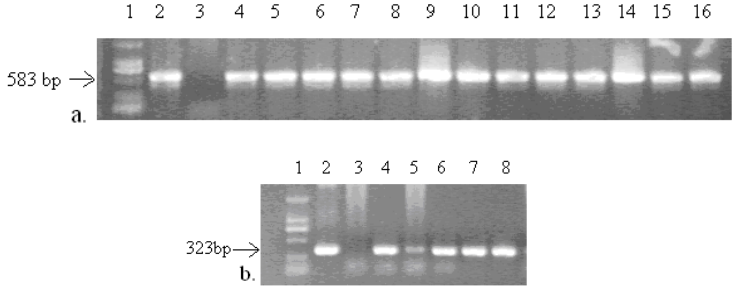

Fig. 3 PCR analysis of transgenic rapeseed

Fig. 3a. Detection of *Hpt* gene. Lane 1, 2, 3, was DNA marker and plasmid control, non-transgenic plant, respectively. Lanes 4-16 were transgenic plants.

Fig. 3b. Detection of DsTI1. Lane 1, 2, 3 was DNA marker and plasmid control, non-transgenic plant, respectively. Lanes 4-8 were transgenic plants.

To test the gene expression in transgenic plants, total RNA was extracted for RT-PCR analysis, from PCR positive transformants and non-transgenic plant. RT-PCR results (Fig. 4) showed that predicted product was observed as a single light band in transformants. 13 positive expressing transformants were obtained finally, resulting in transformation efficiency of 1.4%. Thus, it can be concluded that *DsTI1* gene was integrated into rapeseed genome and expressed.

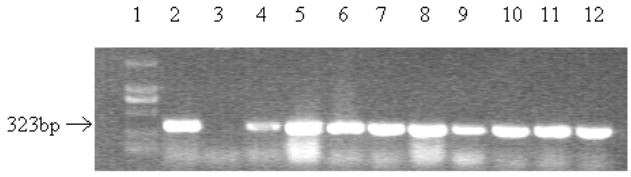

Fig. 4 Detection of *DsTI1* gene transcript by RT-PCR.

Lane 1,2,3 was DNA marker and plasmid control, non-transgenic plant, respectively; lanes 4-12 were transgenic plants

2.4 Insect bioassay results

Transgenic and untransformed leaves were used to rear larvae, and the growth of larval was monitored. Feeding with leaves from non-transgenic regenerated plants as control and *DsTI1* expressing transgenic plants, larvae showed a significant difference between them. In comparison with the control, development delays were observed in larvae that fed on transgenic leaves. And larval weights and sizes were significantly reduced (Fig. 5). These results indicated that presence of DsTI1 in transformant leaves had significantly interfered with several aspects of insect development. A higher mortality was also observed in larvae fed on leaves from *DsTI1*-expressing plants.

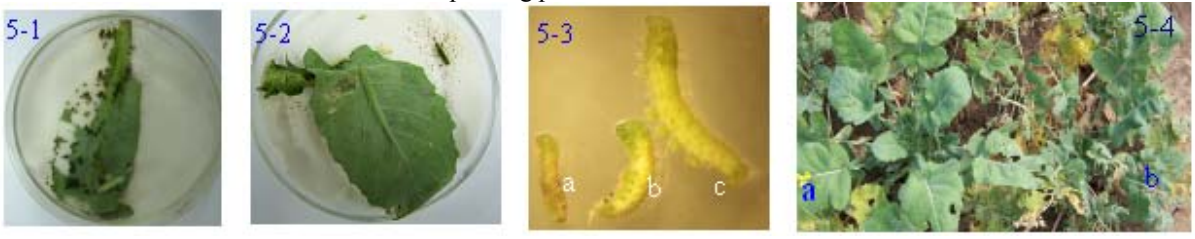

Fig. 5 Insect bioassay on leaves of primary transformants and control with *Pieris rapae* L. larvae.

Fig 5-1, 2, represents control and transformant, respectively.

Fig 5-3. Effects of DSTI1 on *Pieris rapae.* L larvae. Transgenic rapeseed (a, b) and the control (c), respectively.

Fig 5-4. Comparison of transgenic plant (a) and the control (b).

3 Discussion

Sequence alignment revealed that DsTI1 was highly similar to ATTI2, MTI-2, and RTI. The *DsTI1* gene was integrated into rapeseed by *Agrobacterium tumefaciens*-mediated transformation, and increased prominently inhibitory activity to trypsin and chymotrypsin according to the inhibition assays (data not listed here). Preliminary results of insect bioassay also indicated its enhanced insect-resistance in rapeseed.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 30370902)

4 References

[1] Alma, B., Massimo, C., Miriam, O., Valentina, R., Gianni, A., Aldo, T., Daniela, C. (2004) A trypsin inhibitor cDNA from a novel source, snail medic (*Medicago scutellata* L.): cloning and functional expression in response to wounding, herbivore,jasmonic and salicylic acid. Plant Science 167: 337-346

[2] Ryan, C.A. (1990) Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. Ann. Rev. Phytol 28: 425–449

[3] Wang H.Y., Huang Y.C., Chen S.F. and Yeh K.W (2003) Molecular cloning, characterization and gene expression of a water deficiency and chilling induced proteinase inhibitor I gene family from sweet potato (Ipomoea batatas Lam.) leaves, Plant Science 165: 191-203

[4] Octávio L. F., Roseane C. dos S., João A.N. Batista, Ana C. M. Mendesa, Marcus Aurélio M.de Araú joc, Rose G.Monnerata, Maria Fátima Grossi-de-Sá, Sonia M.de Freitas, (2003) Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of *Anthonomus grandis* Phytochemistry 63: 343–349

[5] Sutherland P.W., Burgess E.P.J., Philip B.A., McManus M.T., L.Watson, J.T. Christeller, (2002) Ultrastructural changes to the midgets of the black field cricket (Teleogryllus commodus) following ingestion of potato protease inhibitor II, J. Insect Physiol 48: 327–336

[6] Clauss M. J., Mitchell-Olds T. (2004) Functinal divergence in tandemly duplicated Arabodopsis thaliana trypsin inhibitor genes [J], Genetics 166: 1419-1436

[7] Sambrook J., Russell D.W. (2001) Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press

[8] Junghans H., Metzlaff M. (1990) A simple and rapid method for the preparation of total plant DNA, Biotechniques 8: 176-180

[9] Menegatti E, Tedeschi G, Ronchi S,*et al*. (1992) Purification, inhibitory properties and amino acid sequence of a new serine proteinase inhibitor from white mustard seeds[J]. FEBS Lett.301: 10-14

[10] Ceciliani, F., Bortolotti, F., Menegatti,E., *et al*. (1994) Purification, inhibitory properties, amino acid sequence and identification of the reactive site of a new serine proteinase from oil-rape seeds[J]. FEBS Lett. 342:221-224