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Full Length Research Paper

A plant defensin gene from *Orychophragmus violaceus* can improve *Brassica napus*' resistance to *Sclerotinia sclerotiorum*

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A plant defensin gene, named as *Ovd*, was cloned from *Orychophragmus violaceus* (L.) O. E. Schulz and subsequently introduced into *Brassica napus* genome by *Agrobacterium tumefaciens*-mediated transformation method in sense and antisense forms under the control of the CaMV 35S promoter. Genomic PCR analysis confirmed the integration of the sense and antisense *Ovd* into the host genome. Quantitative RT-PCR showed that the expression of *Ovd* in the sense plant line was stronger than non-transformed plant and antisense plant. The lesion size on detached leaves of the transgenic plants and nontransformed control caused by *Sclerotinia sclerotiorum* mycelia was examined. Lesion size was reduced in sense transgenic plants compared to nontransformed control (15 - 20% reduction area), while it was enlarged in antisense transgenic plants compared to nontransformed control (14 - 20% enlargement). The results showed that the over-expressing transgenic *B. napus* had higher resistance to *S. sclerotiorum*.

Key words: *Brassica napus*, *Orychophragmus violaceus*, plant defensin, *Sclerotinia sclerotiorum*, transformation.

INTRODUCTION

Plant defensins are a class of antimicrobial cysteine-rich peptides whose structural and functional properties resemble those of insect and mammalian defensins (Broekaert et al., 1995). They also can protect seeds, seedlings and plants from attacking of soil-bore pathogens (Terras et al., 1995). Some studies have demonstrated transgenic expression of plant defensins can result in the enhanced resistance of host plants against pathogen (Terras et al., 1995; Lai et al., 2002; Gao et al., 2000; Koike et al., 2002). Furthermore, inhibition of the growth of a broad range of fungi (pathogen) by plant defensins has no concomitant of toxicity to either mammalian or plant cells (Thomma et al., 2002). Therefore, plant defensins are not only important components of host defense, but also can be used to generate transgenic crops with improved pathogen resistance (Thomma et al., 2002).

Brassica napus is one of the most important oilseed crop in many countries, ranking third only to soybean and palm oil in global production. A great deal of effort has gone into improving the quality and disease resistances of *B. napus* using both classical breeding and biotechnological techniques. The plant pathogenic fungus *Sclerotinia sclerotiorum* is an ubiquitous inhabitant of soils in many parts of the world (Boland and Hall, 1994). In China, it is the first pathogen of oilseed. It causes stem rot on oilseed rape (*B. napus*) and leads serious losses in yield every year in the middle and low drainage areas of Yangtse River (Zhang et al., 2003). So it is urgent to improve resistance of the oilseed.

Orychophragmus violaceus (L.) O. E. Schulz, cultivated as an ornamental plant in China, is a valuable oil-seed resource of Cruciferae with high amounts of oleic (20.32%), linoleic (53.17%) and palmitic (14.31%) acids and low amount of linolenic (4.76%) and erucic (0.94%) acids in its seed oil (Luo et al., 1994). *O. violaceus* has a strong disease and insect resistance (Luo et al., 1995). In

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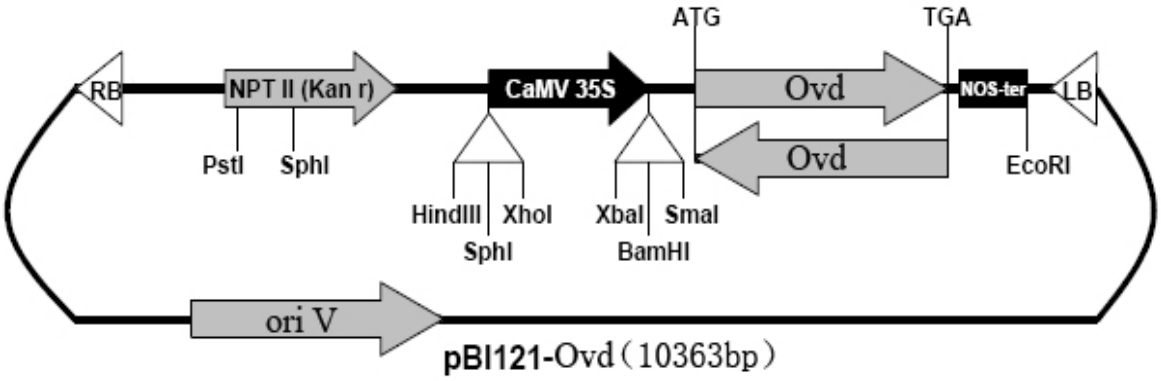

Figure 1. Schematic presentation of cloning of pBI121-*Ovd* recombinant expression plasmid. Plasmid pBI121 was digested with *Sma*I and *Ecl*136II to remove the β-glucuronidase gene and was ligated with the phosphorylated *Ovd* ORF fragment. For the flat-end ligation, two insert directions of (sense and antisense) *Ovd* fragment were produced. RB and LB were the T-DNA borders. Ori V was the replicating origin.

this paper, we describe the molecular cloning of a plant defensin coding sequence from germinating *O. violaceus* seeds and transformation of the cloned gene into *B. napus*. The over-expressing transgenic plants showed higher resistance to *S. sclerotiorum*.

MATERIALS AND METHODS

RNA isolation and cloning of the plant defensin gene (*Ovd*)

O. violaceus seeds were germinated on MS medium. Total RNA was extracted from the germinating seeds at 3 d using the RNA extraction Kit (TIANGEN, P. R. China). The cDNA synthesis was performed with the RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Canada). The full coding sequence of defensin gene was cloned using the primers P1: 5′-ATGGCTAAGTTT GCTTCC-3′ (sense) and P2: 5′- TTAACATGGGAAATAACAGATAC -3′ (antisense). PCR was performed under the following condition: 94°C for 30 s, 50°C for 40 s and 72°C for 30 s. The PCR product was purified and then cloned into pMD18-T vector (TaKaRa Biotechnology Co. Dalian, P. R. China) for sequencing. The resulted plasmid was named pMD18-T-Ovd.

Comparative and bioinformatic analyses

Comparative and bioinformatic analyses of Ovd were carried out online at the websites (http://www.ncbi.nlm.nih.gov). The ORF sequence and deduced amino acid sequence were analyzed and the sequence comparison was conducted through database search using BLAST program (http://www.ncbi.nlm.nih.gov). The signal peptide was predicted with SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/). The phylogenetic analysis of Ovd and the plant defensins from other species was aligned with MEGA4.1 using default parameters. Phylogenetic tree was constructed using MEGA version 4.1 from CLUSTAL X 2.0 alignments. The neighbor-joining method was used to construct the tree.

Agrobacterium tumefaciens strain, plasmid and culture

The *Ovd* ORF fragment was amplified from pMD18-T-Ovd using the above primers with Pfu DNA Polymerase (TaKaRa Biotechnology Co. Dalian，P. R. China) and phosphorylated. Plasmid pBI121 (Chen et al., 2003) was digested with *Sma*I and *Ecl*136II to remove the β-glucuronidase gene and was ligated with the phosphorylated *Ovd* ORF fragment. The constructed vectors were designated as sense and antisense respectively (Figure 1). Then the recombinant plasmids were introduced into *Agrobacterium tumefaciens* (strain EHA105).

A single colony of *A. tumefaciens* was inoculated into 50 ml of liquid LB medium containing 20 mg/l streptomycin, 50 mg/l kanamycin and 40 mg/l rifampicin in an Erlenmeyer flask and shaken at 220 rpm overnight in the dark at 28℃. 10 ml of bacterial suspension were pelleted and resuspended in 250 ml liquid LB medium containing 20 mg/l streptomycin, 50 mg/l kanamycin and 40 mg/l rifampicin, cultured at 28℃ at 250 rpm in the dark until the OD_{600} reached 0.6 - 0.8. For transformation, the culture was centrifuged at 5000 rpm for 5 min and the pellets were resuspended in 250 ml MS liquid medium (pH 5.6) with 19.62 mg/l acetosyringone (Sigma, USA). The bacteria suspension was cultured in the darkness at 120 rpm at 28℃ for an hour before infection of plant cells.

Plant material and culture condition

Seeds of *B. napus* line 84100-18 presented by Professor Mao-lin Wang which had low resistance to *S. sclerotiorum* were rinsed in 70% (v/v) ethanol for 1 min, then surface-sterilized for 12 min in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution. After that, the seeds were rinsed four times in ddH_2O and germinated on MS medium (Murashige and Skoog, 1962) in the darkness for 2 d followed by a light intensity (1600 lux) for an additional 3 - 4 d at 25 ± 2℃ and a 16-h day photoperiod. Hypocotyls were excised and used as explants.

All media were supplemented with 30 g/l sucrose and readjusted to pH 5.8 with 1 M NaOH before autoclaving. 6-benzylaminopurine (BA), 2, 4-D, NAA (a-naphthaleneacetic acid) were added just before autoclaving. Silver nitrate ($AgNO_3$), acetosyringone and antibiotics were filter sterilized with a 0.2 μm membrane and added into the autoclaved media. 0.8% (w/v) agar was added to solidify the media. The media for plants were summarized in Table 1.

Optimization the concentration of kanamycin

Sensitivity of the hypocotyl explants to kanamycin was determined

Table 1. Media for plants in the experiments.

Medium	Composition	Function of media
I	MS	Seeds germination
II	MS+2 mg/l BA+1 mg/l 2,4-D+2.5 mg/l $AgNO_3$+19.62 mg/l acetosyringone	Pre-culture and co-culture media
K0	MS+2 mg/l BA+2.5 mg/l $AgNO_3$	Media K0-K6 for selectiing a suitable concentration of kanamycin
K1	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+5 mg/l kanamycin	
K2	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+10 mg/l kanamycin	
K3	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+15 mg/l kanamycin	
K4	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+20 mg/l kanamycin	
K5	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+25 mg/l kanamycin	
K6	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+30 mg/l kanamycin	
III	MS+2 mg/l BA+2.5 mg/l $AgNO_3$+500 mg/l carbenicillin+10 mg/l kanamycin	Selection media
IV	1/2 MS+0.15 mg/l NAA+250 mg/l cefotaxime	Rooting and propagation media

All media were supplemented with 30 g/l sucrose and readjusted to pH 5.8 with 1 M NaOH before autoclaving. Silver nitrate ($AgNO_3$), acetosyringone and antibiotics were filter sterilized with a 0.2 µm membrane and added into the autoclaved media. 0.8% (w/v) agar was added to solidify the media.

by culture the explants on the media with different concentrations of kanamycin. Hypocotyls from the sterile seedlings were cut into about 1 cm segments and pre-cultured for 2 d on medium II, then transferred to fresh regeneration media K0-K6 at 10-day intervals (Table 1). The experiment was repeated three times with 50 hypocotyl segments per treatment.

Transformation, selection and plant regeneration

The *A. tumefaciens*-mediated transformation procedure in *B. napus* was referred to the published results (Cardoza and Stewart, 2003; De Block et al., 1989; Fry et al., 1987; Moloney et al., 1989). Five to six days after sowing, hypocotyls from the sterile seedlings were cut into about 1 cm segments and pre-cultured for 2 d on medium II. Then the explant segments were immersed in the activated *A. tumefaciens* suspension (OD_{600} = 0.6 - 0.8) for 1 min with slightly shaking. The immersed hypocotyls were patted dry on sterile filter paper, then co-cultured for 2 days on medium II without kanamycin in the dark.

After 2 days of cocultivation, the explants were transferred to medium III containing 10 mg/l kanamycin and 500 mg/l carbenicillin. The explants were subcultured at 10-day intervals to fresh medium of the same composition. The small shoots were formed after 2 - 4 weeks. The healthy shoots (2 cm or longer) were removed from the hypocotyl explants and transferred directly to rooting medium IV. Rooted shoots were propagated with either the top shoot or stem pieces with an axial knob on medium IV or transferred directly to the greenhouse.

PCR analysis of the transgenic plants

DNA was isolated from leaves according to CTAB method (Doyle and Doyle, 1990). PCR analysis of putative transgenic shoots was performed to verify the plant defensin gene transformed into *B. napus*. Primer sets used were: CaMV 35S promoter specific sense primer (5′-GACTAGTGCAAGACCCTTCCTC-3′) coupled with *Ovd* sense primer P1 or antisense primer P2. PCR was performed with 30 cycles at 94℃ for 40 s, 50℃ for 45 s and 72℃ for 30 s. All reactions were preceded by a primary denaturation step at 94℃ for 5 min. Amplified DNA was separated on 1.5% (w/v) agarose gel. Non-transgenic plant was used as the negative control and *A. tumefaciens* with vector pBI121 harboring sense and antisense *Ovd* with CaMV 35S promoter fragment were used as positive control.

Expression analysis of *Ovd* by quantitative RT-PCR

Total RNA was isolated from collected leaves of the sense plant lines (s6, s8 and s12), non-transformed plant and antisense plant line (a3, a4 and a7) selected randomly, using the RNA extraction Kit (TIANGEN, P. R. China). The cDNA synthesis was performed with the RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Canada). Real-time PCR was performed using the Light-Cycler Quick System 350S (Roche Diagnostics K.K.) with SYBR Premix Ex Taq (Takara, China). Each PCR reaction contained 1 × SYBR Premix Ex Taq, 0.2 µM of each primer and 2 µl of a 1:5 dilution of the cDNA in a final volume of 20 µl. The following PCR program was used: initial denaturation, 95℃, 60 s; PCR, 40 cycles of 95℃, 10 s, 57℃, 15 s, 72℃, 15 s. In melting curve analysis, PCR reactions were denatured at 95℃, reannealed at 55℃, then a monitored release of intercalator from PCR products or primer dimmers by an increase to 95℃ with a temperature transition rate of 0.1℃ s^{-1}. To create a standard curve, homologous standards for each gene were used as external standards in all experiments. cDNA quantities were calculated by the second derivative maximum methods of Light-Cycler Software Ver.3.5 (Roche Diagnostics) and all quantifications were normalized using β-actin mRNA as an internal control. The primers were used as follows: *Ovd* specific (forward 5'-TTTCTGCTTTCGAGGCACCAAC-3', reverse 5'-TGATACAGAA GGGACGAGTGTTCAC-3'), actin specific (forward 5'-GTGGGGAT GGAAGCTCCTG-3', reverse 5'-GTG ATCTCTTTGCTCATACGGTC -3').

Fungal resistance bioassays

Ten leaves excised from each plant line were placed into a box bedded with wet-paper and subsequently inoculated with the mycelial agar plug in a diameter of 5-mm cultured from this fungus separately. Plugs were placed on the adaxial surface, near the midvein. The boxes were covered with plastic film and then kept at 20℃ in dark. The lesion diameter was measured after inoculation at 48, 72 and 96 h to evaluate the level of resistance. The results were analyzed with SPSS 13.0 statistic analysis software (SPSS Inc., USA).

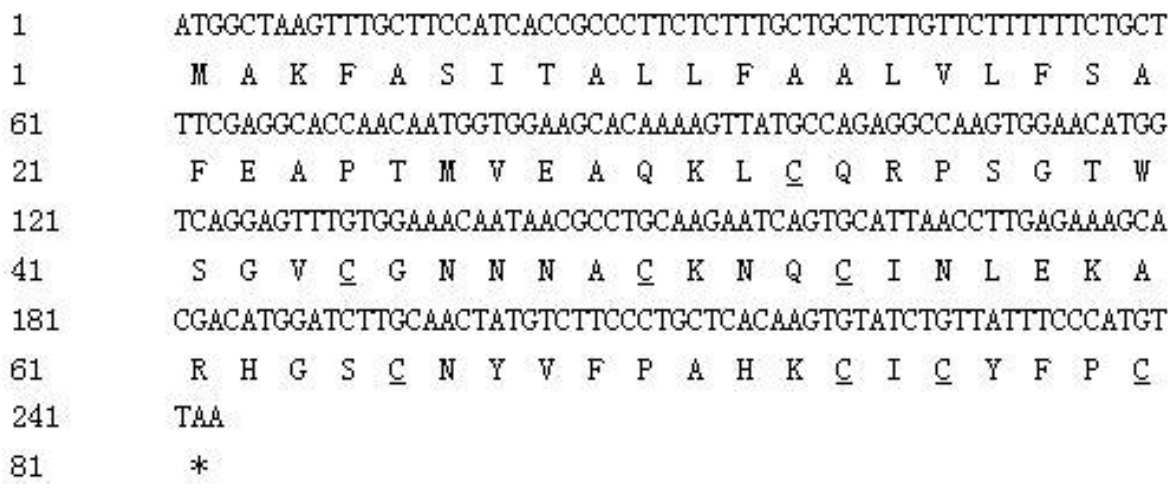

Figure 2. Nucleotide acid sequence and deduced amino acid sequence of *Ovd*. Eight cysteines are underlined.

RESULTS AND DISCUSSION

Clone, comparative and bioinformatic analyses of a plant defensin gene from *O. violaceus*

A pair of primers was designed according to the plant defensin gene sequence of *B. napus* (GeneBank U59459) and used to amplify its homologous gene in cDNA of *O. violaceus'* seeds. The cloned gene is named as *Ovd*. The obtained sequence was 243 bp in length and encoded a putative pre-protein of 80 amino acids (Figure 2). Its forecasted molecular weight is 9 kDa. The richest amino acid residue was Ala (13.8%), followed by Cys (10%). The first 87 bp DNA sequence encodes 29 residues signal peptide analyzed with SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/). The 29-amino acid signal peptide with the signal peptide cleavage site between A_{29} and Q_{30} was identified from the *Ovd* full-length cDNA sequence, which was consistent with the signal peptide cleavage site of Rs-AFP1 (*Raphanus sativus* antifungal protein 1) and Rs-AFP2 (*R. sativus* antifungal protein 2) (Terras et al., 1995). The mature protein was 51 aa in length with a molecular weight of 5.8 kDa. It included 8 Cys that may form four structure-stabilizing disulfide bridges as reported before which were strictly conserved in plant defensins (Broekaert et al., 1995; Lay and Anderson, 2005). A glycine (position 13, according to the mature protein), a serine (position 8), an aromatic residue (position 11) and a glutamic acid (position 29) were also conserved as reported by Lay and Anderson (2005). In comparison of the cDNA sequence of *Ovd* with the sequences of other AFP genes in the NCBI database using BLAST search program，it was found that it has 93% identity with *Rs-AFP1*，90% with *Rs-AFP2* and *Sa-AFP* (*Sinapis alba* antifungal protein gene), and 89% with *Bn-AFP* (*B. napus* antifungal protein gene). The amino acid sequence alignment showed the high identity of Ovd with AFPs in the NCBI database, for example 96% identity with Rs-AFP1, 91% with Rs-AFP2, 91% with *Brassica oleracea* defensin, 88% with Sa-AFP, and 87% with *Arabidopsis thaliana* putative plant defensin PDF1.1 (data not shown). Sequence alignment using DNAMAN also showed the mature protein of Ovd had highly similar with other plant defensins in Brassicaceae (Figure 3). The cDNA sequence has been submitted to GeneBank (GeneBank FJ489240).

Molecular evolution analysis

Ovd was the first defensin gene cloned from *O. violaceus* plant. And it had highly similar with other plant defensins in Brassicaceae. Therefore it would be interesting to investigate its evolutionary position among the phylogenetic tree of various plant defensins. Using MEGA version 4.1 from CLUSTAL X 2.0 alignments, a phylogenetic tree of plant defensins in Brassicaceae was constructed. According to the phylogenetic tree, Ovd had higher identity with Rs-AFP1, Rs-AFP2, *B. oleracea* defensin and *Brassica juncea* defensin (Figure 4), which was closely related to these defensins. Apparently, all the plant defensins in Brassicaceae are derived from a common ancestor in evolution, suggesting that they share a common evolutionary origin. All the analysis results strongly suggest that Ovd is a plant defensin.

Transgenic plants obtained by the optimized transformation and regeneration procedure

To study the function of *Ovd*, the sense and antisense *Ovd* ORF fragments were ligated into pBI121 vector to make the over expression and inhibiting expression vector, respectively. The pBI121- *Ovd* sense and pBI121-*Ovd*

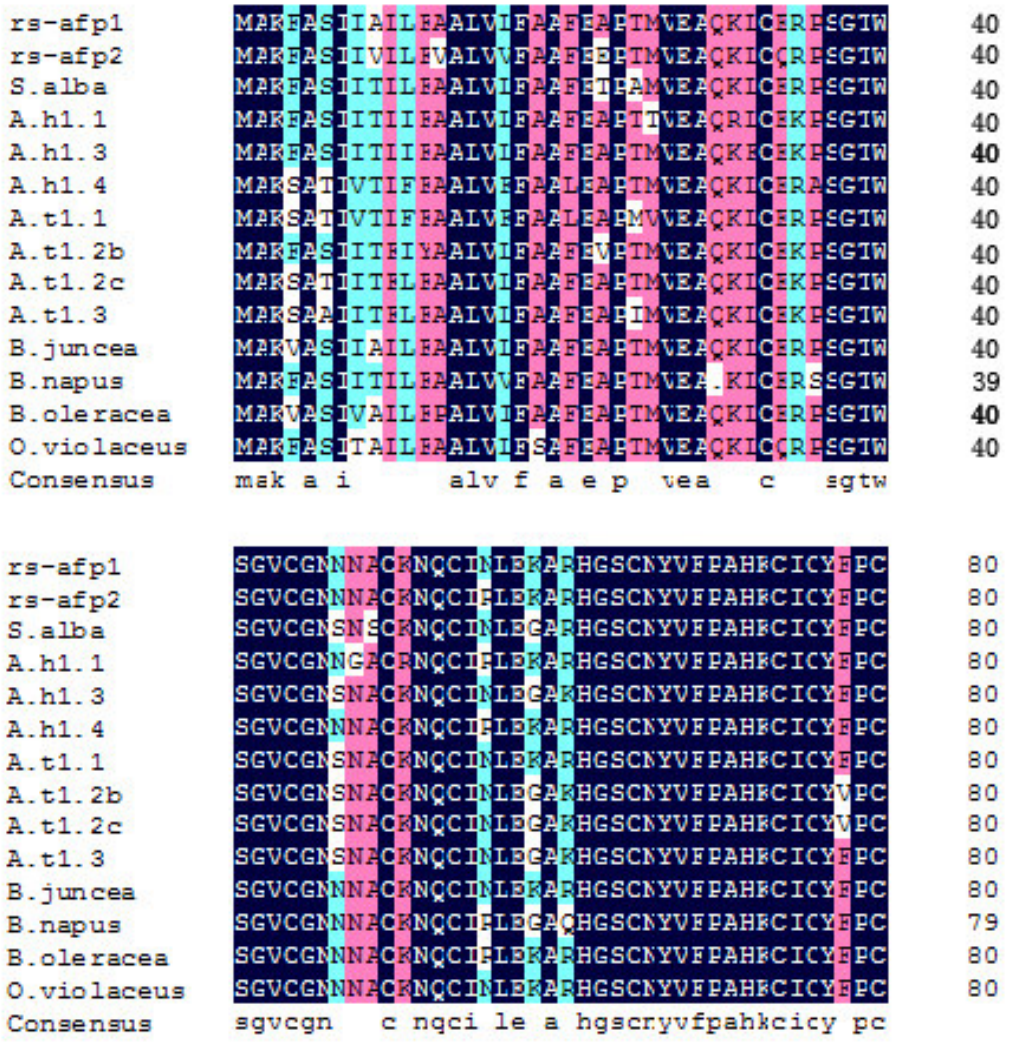

Figure 3. Alignment of Ovd with other plant defensins from Brassicaceae plants. Conserved amino acid residues in all the sequences used in this alignment are black boxed, while similar amino acids are in other colour boxes. Alignments were performed with the DNAMAN program. The plant defensins proteins used in alignment were those from *Raphanus sativus* (GeneBank accession No.ABJ09663.1 and ABJ09664.1), *Sinapis alba* (GeneBank accession No.AAY15221.1), *Arabidopsis halleri* (GenBank accession No. AAY27736.1, AAY27738.1, AAY27739.1), *A. thaliana* (GeneBank accession No. NP_565119.1, NP_180172.1, NP_199256.1, NP_180171.1), *Brassica juncea* (GenBank accession No.ABB59548.1), *B. napus* (GeneBank accession No.AAB03224.1), *B. oleracea* (GeneBank accession No. CAC37558.1).

antisense vector were transformed into *A. tumefaciens* (strain EHA105). Since the hypocotyls explants of *B. napus* line 84100-18 were sensitive to kanamycin and 10 mg/l kanamycin in the media was enough to inhibit normal green shoot differentiation, this concentration was set as selection for transformation.

The hypocotyl explants of seedlings of 5-6d were pre-cultured for 2 d on the medium applied with 1 mg/l 2, 4-D, 2.5 mg/l $AgNO_3$ and 19.62 mg/l acetosyringone (Table 1). After 2 d pre-cultivation, the explants had no overgrowth of the callus and cells were in the meristematic state which was helpful to transformation and regeneration. Then hypocotyls were dipped into bacterium solution and cultured on the same medium as the pre-cultivation medium. After two days co-cultivation they were transferred to medium III with 2 mg/l 6-BA, 2.5 mg/l $AgNO_3$, 500 mg/l carbenicillin and 10 mg/l kanamycin for organogenesis and selection. About 2 - 4 weeks, green shoot grew out from the ends of hypocotyl segments. These shoots rooted on medium IV after 2 - 4 weeks. The successful incorporation of the transgene was verified by the genomic PCR (Figure 5). No positive result occurred with DNA isolated from control plants (non-transformed). Of random selected 13 independent sense kanamycin

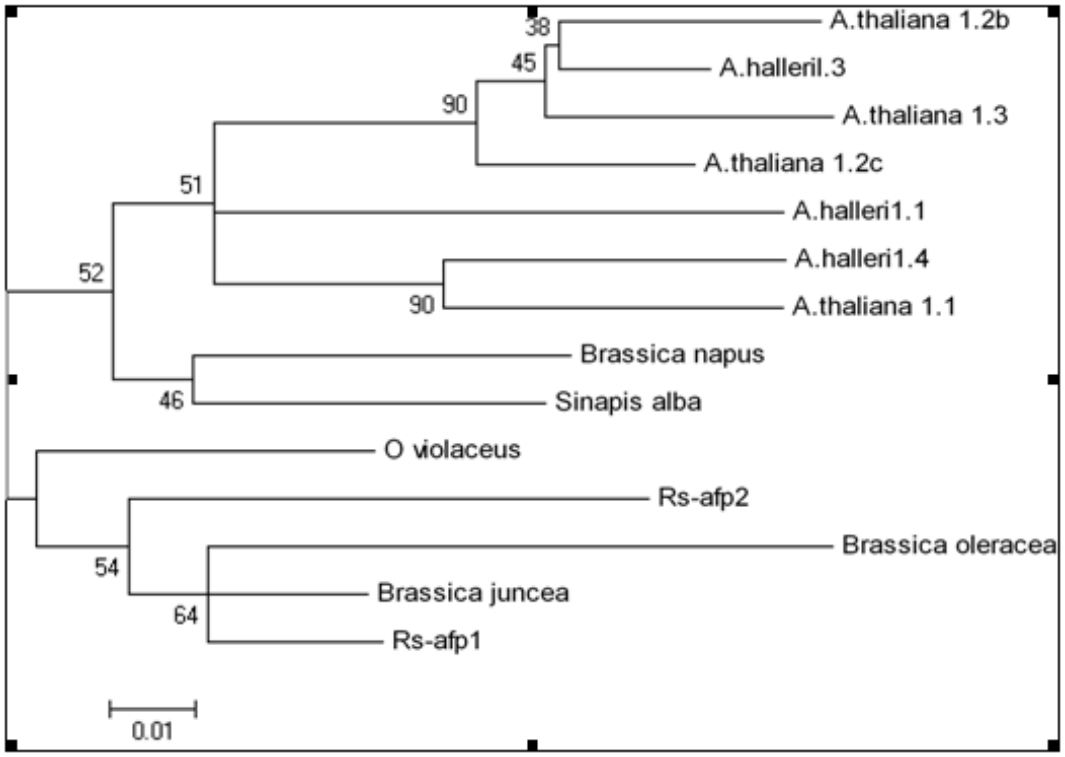

Figure 4. Phylogenetic analysis of plant defensins from *O. violaceus* and other plants in Brassicaceae by MEGA version 4.1 from CLUSTAL X 2.0 alignments. The neighbor-joining method was used to construct the tree. The resources of data were the same with those of Figure 3 (Figure 3).

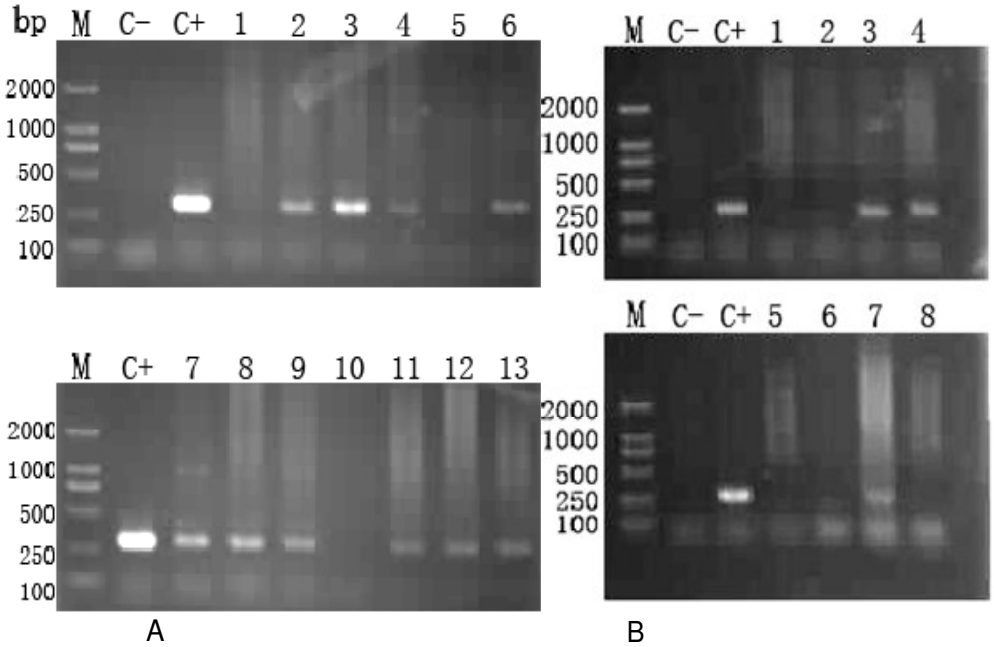

Figure 5. Genomic PCR of transgenic plants. (A) M, DNA molecular weight marker DL2000; C-, Negative control with DNA of the non-transformed plant; C+, Positive control with *Agrobacterium tumefaciens* with pBI121-*Ovd* sense; Lanes 1-13, PCR of different transgenic plants with primers CaMV 35S and *Ovd* antisense. (B) M, DNA molecular weight marker DL2000; C-, Negative control with DNA of the non-transformed plant; C+, control with *A. tumefaciens* with pBI121-Ovd antisense; Lanes 1-8: PCR of different transgenic plants with primers CaMV 35S and *Ovd* sense.

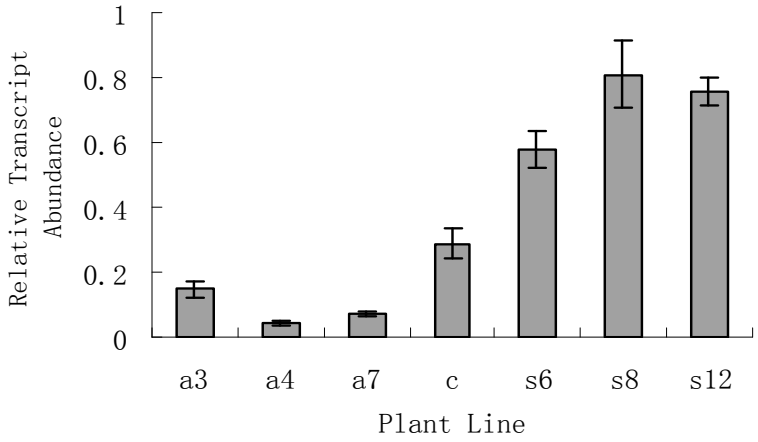

Figure 6. Analysis of the expression of *Ovd* by quantitative RT-PCR. Among the sense plant line (s6, s8 and s12), non-transformed plant and antisense plant line (a3, a4 and a7), the expression level of *Ovd* was the highest in the sense plant line. On the other hand this also verified *Ovd* has been transformed into *B. napus*. Data were from three biological replicates with SE shown as error bars.

Table 2. Comparative effect of *S. sclerotiorum* attack on transgenic and wild-type *B. napus* leaves.

Plant line	48 h	P value	72 h	P value	96 h	P value
a3	1.94 ± 0.12	0.007	3.62 ± 0.36	0.038	5.49 ± 0.38	0.048
a4	1.98 ± 0.13	0.002	3.65 ± 0.35	0.023	5.52 ± 0.44	0.045
a7	1.95 ± 0.13	0.006	3.66 ± 0.38	0.025	5.57 ± 0.46	0.030
c	1.78 ± 0.14		3.36 ± 0.24		5.17 ± 0.43	
s6	1.62 ± 0.15	0.012	3.13 ± 0.11	0.006	4.82 ± 0.39	0.037
s8	1.57 ± 0.06	0.000	3.02 ± 0.15	0.001	4.68 ± 0.44	0.011
s12	1.60 ± 0.08	0.001	3.06 ± 0.13	0.002	4.79 ± 0.39	0.027

Fungal resistance assays were performed using detached leaves inoculated with an agar plug of *S. sclerotiorum* mycelia from the actively growing edge of a fungal culture. The lesion diameter was measured after inoculation at 48, 72 and 96 h to evaluate the level of resistance. Plants of s6, s8 and s12 were transformed plants with *Ovd* sense. Plants of a3, a4 and a7 were the transformed plants with *Ovd* antisense. The control was a non-transgenic plant. The average value and *P* value were obtained by computing data from ten leaves in each plant with SPSS 13.0. Comparing the lesion diameters, those of sense transgenic plants were the shortest and lesion diameters of antisense transgenic plants were the longest. Comparing with the non-transformed plant, the difference of the lesion diameters of transformed plants reached significance level ($p < 0.05$ or 0.01). Means and SE are shown for three replicates.

resistant plants and 8 independent antisense kanamycin resistant plants, 10 and 3 plants had positive band respectively.

Analysis of the expression of *Ovd* was performed by quantitative RT-PCR. The expression values of the individual genes were normalized using the expression level of β-actin as an internal standard. Mean expression values and SE values were calculated from the results of three independent experiments. The result (Figure 6) showed that *Ovd* mRNA was more abundant in sense plant lines than other lines. The expression level of *Ovd* was the highest in the sense transgenic plant line, while the expression level in the antisense plant line was the lowest. On the other hand this also verified *Ovd* has been transformed and expressed in *B. napus*.

Resistance of transgenic plants to *S. sclerotiorum*

To test the resistance to *S. sclerotiorum*, sense plants (s6, s8, s12) and antisense plant (a3, a4, a7) were selected randomly. Fungal resistance assays were performed using detached leaves inoculated with an agar plug of *S. sclerotiorum* mycelia from the actively growing edge of a fungal culture. The lesion diameter was measured after inoculation at 48, 72 and 96 h to evaluate the level of their resistance (Table 2). Comparing the lesion diameters, those of sense transgenic plants were the shortest and

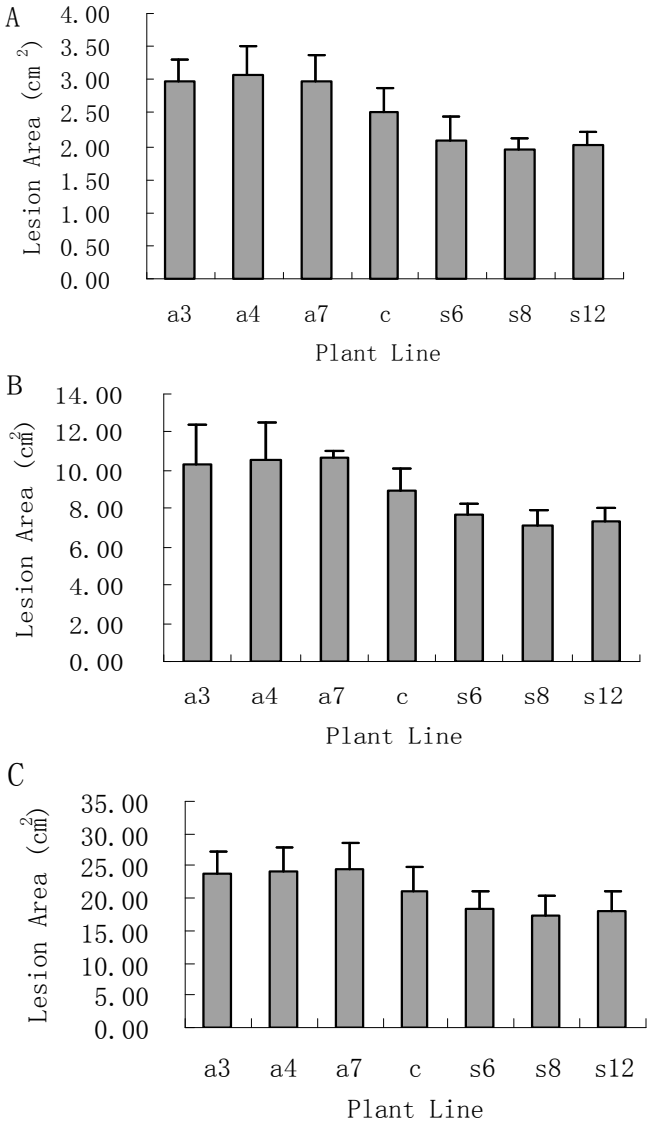

Figure 7. Lesion size (in cm^2) on detached leaves in response to inoculation with *S. sclerotiorum* after 48 h (A), 72 h (B) and 96 h (C). Means and SE are shown for three replicates.

lesion diameters of antisense transgenic plants were the longest. Comparing with the non-transformed plant, the difference of the lesion diameters of transformed plants reached significance level ($p < 0.05$ or 0.01). Lesion size was reduced in sense transgenic plants compared to non-transformed control (15 - 20% reduction), while it was enlarged in antisense transgenic plants compared to non-transformed control (14 - 20% enlargement) (Figure 7). In general, the hierarchy of lesion size was plants with sense *Ovd* < control plants < plants with antisense *Ovd*. This was consistent with the expression level of *Ovd* performed by quantitative RT-PCR. This result showed that to a certain extent, plant defensin *Ovd* can confer enhanced resistance to *S. sclerotiorum*. The line of *S. sclerotiorum* used in our study was highly pathogenic in Chengdu, so it was possible to affect the resistance of transgenic plants.

B. napus is one of the most important oilseed crops in many countries and *S. sclerotiorum* is one of the most serious pathogeny of oilseed. For these reason, a great deal of effort has gone into improving the quality and disease resistances of *B. napus*. Chemical methods have been used to control this disease. However, due to negative environmental effects, they are not a good choice. And there were no rapeseed cultivars found to be immune to *Sclerotinia* (Zhao and Meng, 2003). So it is urgent to improve resistance of the oilseed. The genetic engineering can target to specific characteristics and is thought to be most practical if efficient, genotype-independent and reproducible transformation and regeneration system were available.

Now several plants have been transformed with plant defensin genes. Lay and Anderson (2005) have reviewed these reports. For example, constitutive expression of the radish defensin (Rs-AFP2) enhanced resistance of tobacco plants to the fungal leaf pathogen *Alternaria longipes* and similarly in tomato to *A. solani*. Canola (*Brassica napus*) constitutively expressing a pea defensin had slightly enhanced resistance against blackleg (*Leptosphaeria maculans*) disease. Expression of the alfalfa defensin (alfAFP) in potatoes enhanced their resistance to the fungal pathogen *V. dahliae*. These transgenic plants usually showed the resistance to one pathogen, seldom to several pathogens.

In this paper, we cloned a plant defensin gene (*Ovd*) from *O. violaceus* and transformed *B. napus* with *Ovd* by the *A. tumefaciens* mediated method. The transformed plants with *Ovd* sense showed a higher resistance to *S. sclerotiorum* than non-transgenic plants and the transformed plants with *Ovd* antisense. To our knowledge, this is the first report of improved higher resistance to *S. sclerotiorum* by transformation of *B. napus* with the plant defensin gene (*Ovd*) derived from *O. violaceus*. The obtaining of transgenic *B. napus* with higher resistance to pathogen will improve the crop quality, especially the wider adaptation; consequently the increased output is also expectable. Further studies are on going in our laboratory.

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REFERENCES

Broekaert WF, Terras FR, Cammue BP, Osborn RW (1995). Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 108: 1353-1358.

Cardoza V, Stewart CN (2003). Increased Agrobacterium-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyl segment explants. Plant Cell Rep. 21(6): 599-604.

Chen PY, Wang CK, Soong SC, To KY (2003). Complete sequence of

the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed. 11: 287-293.
De Block M, De Brouwer D, Tenning P (1989). Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. 91: 694-701.
Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissues. Focus, 12: 13-15.
Fry J, Barnason A, Horsch RB (1987). Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. Plant Cell Rep. 6: 321-325.
Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang JH, Rommens CMT (2000). Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. 18: 1307-1310.
Koike M, Okamoto T, Tsuda S, Imai R (2002). A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. Biochem. Biophys. Res. Commun. 298: 46-53.
Lai FM, DeLong C, Mei KF, Wignes T, Fobert PR (2002). Analysis of the DRR230 family of pea defensins: gene expression pattern and evidence of broad host-range antifungal activity. Plant Sci. 163: 855-864.
Lay FT, Anderson MA (2005). Defensins-components of the innate immune system in plants. Curr. Protein Pept. Sci. 6: 85-101.
Luo P, Lan ZQ, Li ZY (1994). *Orychophragmus violaceus*, a potential edible-oil crop. Plant Breed. 113: 83-85.
Luo P, Chen ZL, Lan ZQ (1995). Studies on the plant genetic resource *Orychophragmus violaceus*. Sichuan University Press, pp. 24-27 (in Chinese).
Moloney MM, Walker JM, Sharma KK (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. 8(4): 238-242.
Terras FR, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van Leuven F, Vanderleyden J, Cammue BPA, Broekaert WF (1995). Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell. 7: 573-588.
Thomma BP, Cammue BP, Thevissen K (2002). Plant Defensins. Planta. 216: 193-202.
Zhang ZY, Guan CY (2003). Identification of resistance (tolerance) to *Sclerotinia sclerotiorum* and resistance breeding in rapeseed. Hubei Agricultural Sciences. (3): 38-43 (in Chinese).
Zhao JW, Meng JL (2003). Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.) Theor. Appl. Genet. 106: 759-764.