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Research Article

Overexpression of cysteine protease in transgenic *Brassica rapa* enhances resistance to bacterial soft rot and up-regulate the expression of various stress-regulated genes

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Abstract Cysteine proteases have been known as a critical factor in plant defense mechanisms in pineapple, papaya, or wild fig. Papain or ficin is one kind of cysteine proteases that shows toxic effects to herbivorous insects and pathogenic bacteria. However, resistance to bacterial soft rot of plants genetically engineered with cysteine protease has been little examined thus far. We cloned a cysteine protease cDNA from *Ananas comosus* and introduced the gene into Chinese cabbage (*Brassica rapa*) under the control of the cauliflower mosaic virus 35S promoter. The transgene was stably integrated and actively transcribed in transgenic plants. In comparisons with wild-type plants, the T_2 and T_3 transgenic plants exhibited a significant increase in endo-protease activity in leaves and enhanced resistance to bacterial soft rot. A cDNA microarray analysis revealed that several genes were more abundantly transcribed in the transgenic than in the wild type. These genes encode a glyoxal oxidase, PR-1 protein, PDF1, protein kinase, LTP protein, UBA protein and protease inhibitor. These results suggest an important role for cysteine protease as a signaling regulator in biotic stress signaling pathways, leading to the build-up of defense mechanism to pathogenic bacteria in plants.

Keywords *Brassica rapa*, Cysteine protease, Microarray analysis, Soft rot resistance, Transgenic plant

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Introduction

Cysteine proteases are involved in a variety of proteolytic functions in higher plants (Granell et al. 1998). A number of genes encoding papain-like cysteine proteases have been isolated from leaves (Lohman et al. 1994; Ueda et al. 2000; Gepstein et al. 2003), flowers (Eason et al. 2002), legume nodules (Kardailsky and Brewin 1996), fruit (Muta et al. 1997) and germinating seeds (Ling et al. 2003). Some cysteine proteases have specific characteristics such as a C-terminal KDEL motif. This motif, which is an endoplasmic reticulum retention signal for soluble proteins, allows cysteine protease propeptides to be stored either in a special organelle, called the ricinosome (Schmid et al. 1999), or in KDEL vesicles (KV) before transport to vacuoles through a Golgi complex-independent route (Okamoto et al. 2003).

Cysteine protease expression has been intensively studied with various expression patterns reported for different stages of plant development (Buchanan-Wollaston and Ainsworth 1997; Guerrero et al. 1998; Xu and Chye 1999). Such proteases are involved in processing and degradation of seed storage proteins (Shimada et al. 1994; Toyooka et al. 2000), fruit ripening (Alonso and Granell 1995) as well as in legume nodule development (Naito et al. 2000). They are also implicated in responses to stresses such as wounding, cold, and drought (Schaffer and Fischer 1988; Koizumi et al. 1993; Linthorst et al. 1993; Harrak et al. 2001) as well as in programmed cell death, reproduction, senescence and pathogenesis (Xu and Chye 1999; Solomon et al. 1999; Schmid et al. 1999).

D' Silva et al. (1988) demonstrated that the cleavage of poly (ADP-ribose) polymerase (PARP) by cysteine proteases was activated during the hypersensitive response of cowpea to cowpea rust fungus. PARP is normally a target for cleavage by caspases during apoptosis in animals (Vaux and

Korsmeyer 1999). Inhibition of cysteine proteases with cystatin blocked programmed cell death in soybean triggered by either oxidative stress or an avirulent strain of *Pseudomonas syringae* pv. *glycinea* (Solomon et al. 1999). Both Solomon et al. (1999) and D' Silva et al. (1988) have suggested a regulatory role for cysteine proteases and their inhibitors during the hypersensitive response in plants.
In this paper, we transformed Chinese cabbage (*Brassica rapa*) with a *BAA1* gene encoding cysteine protease from pineapple fruit, with the purpose of comparing the resistance of bacterial soft rot between the transgenic plants and the wild-type (WT) counterpart. Patterns of gene expression in leaves were also compared by microarray analysis.

Materials and methods

Construction of transformation vectors

The cDNA encoding cysteine protease was isolated from the pineapple fruit (designated as *BAA1*) and digested with *Xho*I and *Kpn*I to contain the open reading frame thereof. The *Xba*I−*Kpn*I fragment was cloned between the *Xba*I and *Kpn*I sites of a binary vector pBI121-Hm, which contained the CaMV 35S promoter. The binary vector is a derivative of pBI121 (Clontech) and contains two antibiotic resistance genes, i.e., neomycin phosphotransferase II gene and hygromycin phosphotransferase gene.

Transformation of *Chinese cabbage* (*Brassica rapa*)

The vector containing the sense *35S:BAA1* construct was transferred from *Escherichia coli* JM109 into *Agrobacterium tumefaciens* via tri-parental mating with an *E. coli* strain containing a mobilization plasmid, pRH021. The pBI121-Hm vector containing the sense *35S:BAA1* construct was transferred into *Agrobacterium* LBA4404 strain and transferred to Chinese cabbage (OSOME ecotype) by the *Agrobacterium* method (He 1990). Transgenic seedlings were selected in terms of resistance to both kanamycin and hygromycin in a selection medium. The medium consisted of a full-strength MS medium (Murashige and Skoog 1962), supplemented with 10 g liter^{-1} sucrose, 0.1 g liter^{-1} myoinositol, 0.5 g liter^{-1} MES, 500 mg liter^{-1} carbenicillin, 200 mg liter^{-1} Claforan (Hoechst), 50 mg liter^{-1} kanamycin and 40 mg liter^{-1} hygromycin. After adjusting the pH to 5.7 with KOH, the medium was solidified with 0.5% (w/v) Gelan gum (Sigma). The transgenic T_2 and T_3 plants from independently derived transgenic lines were used in this study.

RT-PCR and realtime PCR analysis

Total RNA was isolated using TRIzol method (Invitrogen). In all cases, RNA was treated with RNase free DNase and the DNase was removed according to the instructions of the supplier (AMBION). The RNA was quantified in a spectrophotometer at 260 nm. Approximately 5 μg of total RNA was reverse transcribed using random hexamers (Amersham Pharmacia Biotech Inc) and reverse transcriptase without RNaseH activity (Fermentas) in a final volume of 20 μL. 2.5 $\times 10^5$ copies of GeneAmplimer pAW 109 RNA (Applied Biosystem) were added to the reverse transcription reaction. The cDNA was diluted 50 times and 5 μL was used for amplification by PCR in a volume of 25 μL. *BAA1* gene-specific primers were used (BAA-F: 5'-ATGGCTTCCA AAGTTCAACTCGTG-3') and (BAA-R: 5'-TCAAGTTT CAGAAACCATCTT-3'). The constitutively expressed actin gene (ACT-F: 5'- ATGGTTGGGATGGGTCAAAAA -3' and ACR-R: 5'- ACGGAGCTCGTTGT AGAAAGT -3') was used as an internal control of RNA quantity and GeneAmplimer pAW 109 RNA (109-F: 5'-CATGTCAAA TTTCACTGCCTTCATC-3' and 109-R: 5'-TGACCACCC AGCCATCCTT-3') as positive control of the RT-PCR efficiency. Primer sequences used for confirm microarray analyses were showed Table 1. In order to get semi-quantitative results, the number of cycles of the PCR reactions was adjusted for each gene to obtain barely visible bands in agarose gels. Aliquots of the PCR reaction were loaded on agarose gels and stained with ethidium bromide.
For real-time quantitative PCR experiments (RT-qPCR), the cDNAs were diluted 100 times, *BAA1* and actin target genes were amplified using gene specific primers designed from the coding sequence using the Primer Express 1.5 software (Applied Biosystems). The primer pairs for *BAA1* were 5'-CTCTATCGGAGCAAGAAGTTCTCG-3' and 5'- GCCAC ACCGTTGTTAGATATG ATG -3' and for actin 5'- ATGG TTGGGATGGGTCAAAAA -3' and 5'- ACGGAGCTCG TTGTAGAAAGT -3'. Both primers pairs amplified 115 bp amplicons with similar melting temperatures (ca. 81℃). Samples were amplified using a SmartCycler II (Takara) detector in conjunction with the Quantitect SYBR green PCR kit (Qiagen GmbH). Thermal cycling conditions were 15 min denaturation at 95℃ followed by 40 cycles (15 sec at 95℃, 30 sec at 58℃, 30 sec at 72℃). Relative expression levels were calculated and the primers validated using the △△CT method (http://www.appliedbiosystems.com) using data obtained with the actin-specific primers as an internal control.

Table 1 Gene-specific PCR primers for semiquantitative RT-PCR amplification

Gene ID	Accession No.[a]	Primer pair	Amplicon size
At1g20760	T42644	F: GGAGCAAGGAGGTAGTGCAG R: TCCCAATCTTCATCCCAAAC	218
At5g19580	NM12196	F: CGATGGAAGACGGAGATGAT R: GGGCTTGAGCTGTCTGAAAC	279
At4g25780	NM118710	F: TGCGTGGATTGGAGAGTACA R: TGCCAGGAGGGAAATAGTTG	272
At1g75830	NM126274	F: TTTCTTTGCTGCTCTTGAAGC R: GTTGCAAGATCCATGTCGTG	334
At2g40560	NM129618	F: TTGTGG ACGAGTCTTCGTTG R: AAGCTTGTTTGAGGCTTGGA	191
At5g56540	AK117330	F: GGAGGCAATGAAGATGAAGC R: GAAGAAT CCAAAGGCCATCA	261
At5g47970	NM124171	F: ATGGTTTGCTGAGAGGGATG R: GGAGCAGCACTAAACCAAGG	132
At2g18840	NM127440	F: ATG GTTTGCTGAGAGGGATG R: GGAGCAGCACTAAACCAAGG	202
At5g38195	NM123180	F: AAGAAGGCATGAAGGAAGCA R: CCC AAGAGTTTGACCACGTT	309
At4g12730	NM117342	F: ATCATGCTAAC GCCAGAACC R: GGGGCATTTAAGTTTGCTGA	305
At2g38870	NM124955	F: GCTTACGGGAACAAATGGTG R: GACGACGATACGGTTTCCAT	309
At1g63730	DQ527168	F: TTTTGCATTCAGCCCCTTAC R: TGCCAAGTTGAAGTGAGTCG	183
At3g54620	AY054645	F: AAAACA AGCTCAAGGCTCCA R: CTGCGTCTCCATCAAGATCA	242
At5g12120	NM121250	F: GCAAAGTCCCTTCTGTGCTC R: TTGGTTT TCGGATTCTCCAC	258
At5g44420	NM123810	F: CACCCTTATCTTCGCTGCTC R: GTTGCATGATCCATGTTTGG	186
At1g64160	NM118500	F: TTTCAAATTCGGGAAACTCG R: CCCTCCGACAACAGACAAAT	150
At1g66270	NM105298	F: AACTTGGGACCACAGATTCG R: CCATCTTGCCATTCGAAGTT	180
At5g21105	NM001161257	F: AACAAACTC ACCACCGAAGG R: CCTTTAGCCACGTCCACAAT	258
At3g07080	BT008641	F: GAGAAGGCTGTTTTGCTTGG R: AACTCGC ATCCTTGTCCAAC	324
At4g12530	NM117323	F: GCTATGAGGCCTTGTTGCTC R: CATCAGGAAGGGTACCTCCA	237

[a] The nucleotide sequences used for designing the primers are shown by their accession number. The designed primer pairs yielded a strong single band for each gene

Immunoblot analysis

Rosette leaves (about 0.2 g FW) were homogenized with a Polytron in 1 mL of ice-chilled buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM DTT and 0.2 mM *p*-mercuribenzoate. The homogenate was centrifuged at 27,000 x g for 20 min at 4℃. Proteins in the supernatant were quantified as described by Bradford (1976) with bovine serum albumin as the standard. Proteins were separated by SDS-polyacrylamide gel electrophoresis using a Ready gel J gel (Bio-Rad) by the method described by Laemmli (1970), and electroblotted onto polyvinylidene difluoride membranes (Sequiblot PVDF membrane, Bio-Rad). Membranes were incubated with a *BAA1* antibody raised in rabbit against a peptide

corresponding to the 14 amino acids (CSYVRSNDESMKYA) of the *BAA1* C-terminus. Immunoreactive proteins were detected with a second antibody, goat anti-rabbit IgG (H+L) (Human IgG Adsorbed) horseradish peroxidase (Bio-Rad).

Protease activity assay

The assessment of total protease activity of Chinese cabbage leaves was carried out according to the method of Holwerda and Rogers (1992). In brief, 50 μL leaf extract was mixed with 300 μL 100 mM sodium acetate buffer (pH 5.0) or 300 μL 100 mM sodium phosphate buffer (pH 7.0) containing 50 μL 0.6% (w/v) azocasein (Sigma), supplemented with 100 μL 0.1% Brij, and the mixture was incubated at 30℃ for 3 h. The reaction was terminated by adding 200 μL 10% TCA, filtered and the absorbance of the filtrate at 366 nm was determined (HITACHI U-2001 spectrophotometer). One unit of protease activity was defined as the enzyme amount that gives 0.01 absorbance increase per min.

Inoculation of soft rot bacteria and resistance evaluation

Ren's (2001) method was used for inoculation of *Pectobacterium carotovorum* ssp. obtained from the Korean Agricultural Culture Collection (KACC, http:mgd.niast.go.kr), National Institute of Agricultural Science & Technology, RDA, Suwon, Korea. The bacteria for inoculation were freshly grown on NA agar medium (5 g of peptone, 3 g of beef extract, 2 g of yeast extract, and 15 g of Bacto-agar per liter) at 28℃. Sterile water (mL) was added to each plate and freshly-cultivated bacteria were scaped gently from the agar medium with a sterile glass rod. After centrifugation at 5,000 x g, the bacterial pellet was resuspended in sterile water and adjusted to $OD_{600} = 0.2$ (2.5×10^8 CFU/mL). The leaves and roots were inoculated with the bacterial suspension by syringe infiltration. The inoculated plants were then transferred to a growth chamber and incubated at 28℃ under continuous light, checked for 12 to 96 h after inoculation. The control plants were treated similarly with sterile water.

cDNA microarray analysis

Total RNA was extracted from leaves with TRIZOL reagent (Life Technologies) according to the manufacturer's instructions and purified by passing through a RNeasy column (Qiagen). After checking the RNA quality by gel electrophoresis (Agilent Bioanalyzer, Agilent Technol.), 40 μg of total RNA was reverse transcribed by using oligo dT12-18 primer and aminoallyl-dUTP. The synthesized cDNA was labeled by reaction with dye (NHS-ester Cy3 or Cy5; Amersham Biosci.) according to the method described by Hughes et al. (2001). The labeled cDNA was applied to the DNA microarray (*Arabidopsis* microarray, Agilent Technol.) and hybridized at 60℃ for 17 h. After washing, the microarray was scanned on a ScanArray 5000 (GSI Lumonics) and the image was analyzed by using QuantArray software (GSI Lumonics). The signal intensity of each spot was calibrated by subtraction of the intensity of the negative control. In analyzing the data, genes with expression levels higher than 500 were taken into account, and the genes with signal intensity ratios in the transgenics vs. the WT above 2.0 and below 0.5 were considered that the expression was up- and down-regulated, respectively, in the transgenics.

Results

Analysis of the 35S:BAA1 plants

The *BAA1* gene encoding cysteine protease was isolated from pineapple fruit by RT-PCR using primers designed against the published sequence (GeneBank: GI2351106). We introduced the *BAA1* gene fused with the CaMV 35S promoter into Chinese cabbage (Fig. 1A). After *Agrobacterium*-mediated transformation, transformants were selected for hygromycin resistance (50 mg/L hygromycin). Transformants started to build up callus after 10 days-culture in the induction medium, an amorphous mass of cells appeared from the callus during the late period of the second generation of the culture, and many multi-shoots appeared from the third generation of the culture. After culture in the regeneration medium until the length of the plantlets reached 7-8 cm, the organisms were induced to form roots (Fig. 1B). The rooted individuals were transferred to the pot to induce flowering by treatment at 8-10℃ for 40 days. The flowered individuals were bud pollinated to produce T_1. The 35S:*BAA1* transgenic T_2 and T_3 lines form the basis of this study. The T_2 seeds were sown in the antibiotics-containing agar medium to eliminate non-transgenic individuals, while the homogeneous T_3 seeds were sown in soil. Total RNA and water-soluble proteins were extracted from rosette leaves of the WT and several T_2 transgenic plants grown in an agar medium for 4 weeks under control conditions as described in Materials and Methods. RT-PCR and realtime PCR analysis showed that a signal corresponding to *BAA1* was detected in the transgenic lines, while no signal was detectable in the WT (Fig. 2A). Protein gel blot hybridization with polyclonal antibody raised against *BAA1* showed that a band with a molecular mass between 36 and 50 kDa was detected that corresponded, in terms of molecular mass, to sweet potato

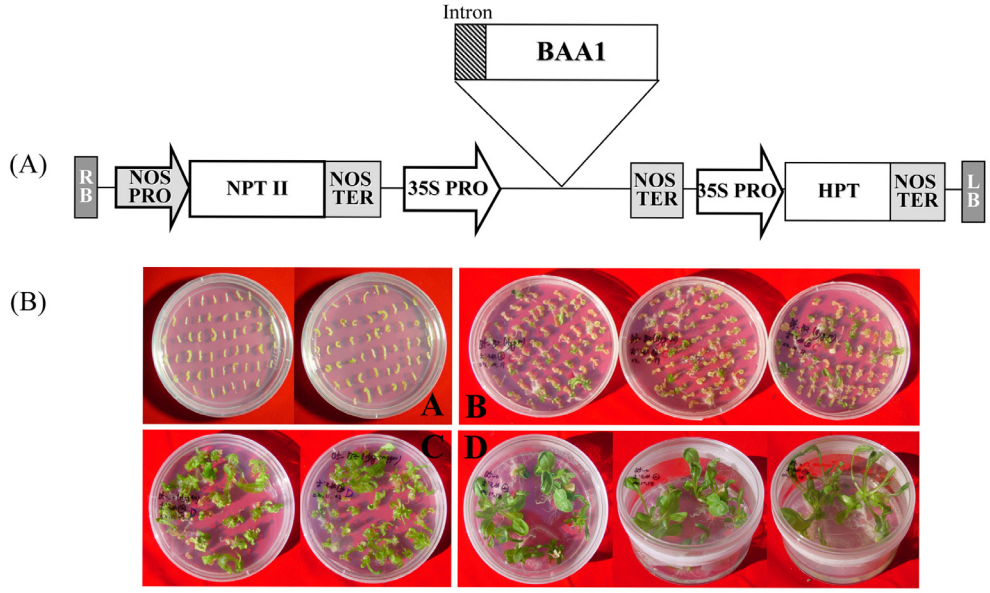

Fig. 1 Vector construction and transformation. (A); Binary plant expression vector pIG 121 Hm for the expression of BAA1. Nos-p, nos gene promoter; HPT, hygromycin phosphotransferase gene; Nos-t, nos gene terminator; 35S-p, Cauliflower mosaic virus 35S- promoter; BAA1; LB, left border; RB, right border. (B); Plant transformation was used to hypocotyles of *B. rapa* by *Agrobateium* method (Material and Methods)

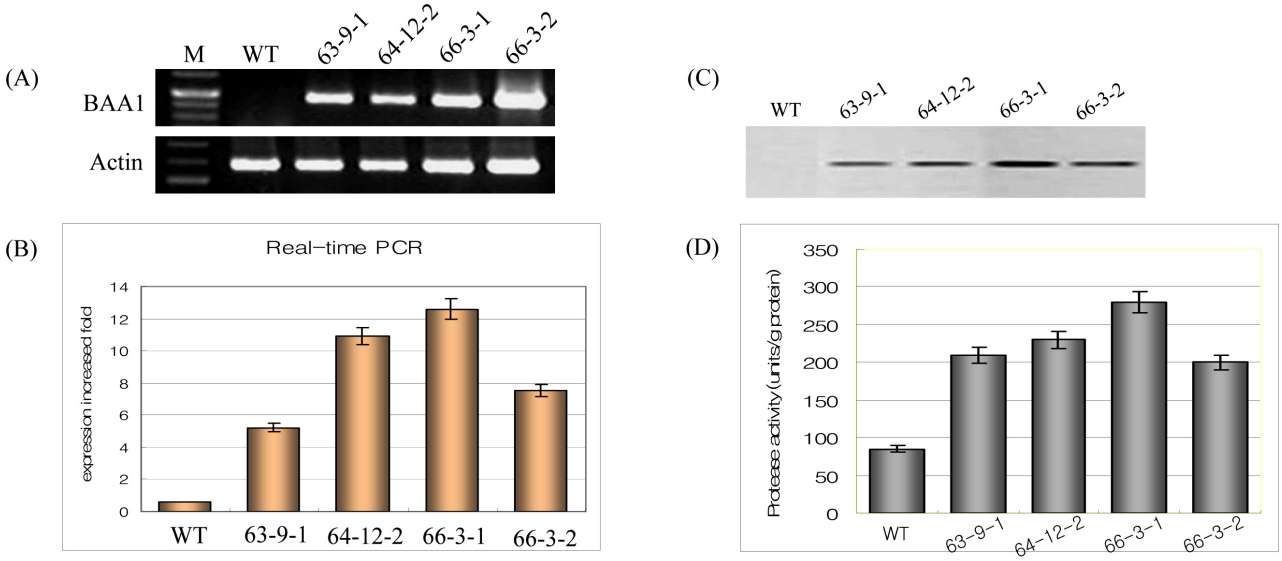

Fig. 2 Expression of BAA1 gene in transgenic plants. (A); The amplification products of the BAAl were separated using a 1.5% Agarose gel. M: DNA-ladder; W: PCR generated from the cDNA template of the wild-type plant; P: PCR product generated from the cDNA template of the pIG 121 Hm plasmid that contains the BAA1: 1~8, PCR products generated from the cDNA template of independent transgenic lines. (B); mRNA expression level using realtime PCR analysis. (C); Western blot analysis of BAA1 protein expression in *Brassica rapa* leaf tissue. Total soluble protein sample (20 μg) from the wild type and two transgenic plants using an antimicrobial BAA polyclonal antibody. (D); Protease activity analysis

SPCP 3 (Chen et al. 2006) (Fig. 2B). In addition, the transgenic lines showed a 5- to 6-fold increase in endo-protease activity in leaves over the WT (Fig. 2C). Since the growth rate under normal conditions did not differ between the WT and transgenic lines, the above results show the actual increase of cysteine protease in the transgenic lines.

Transgenic lines exhibit enhanced soft rot disease resistance

Three transgenic lines (B63-9-1, B64-12-2 and B66-3-1) were selected based on high expression level of *BAA1* gene (Fig. 2A, C). When wild type and transgenic lines were inoculated with *Pectobacterium carotovorum* subsp. *caro-*

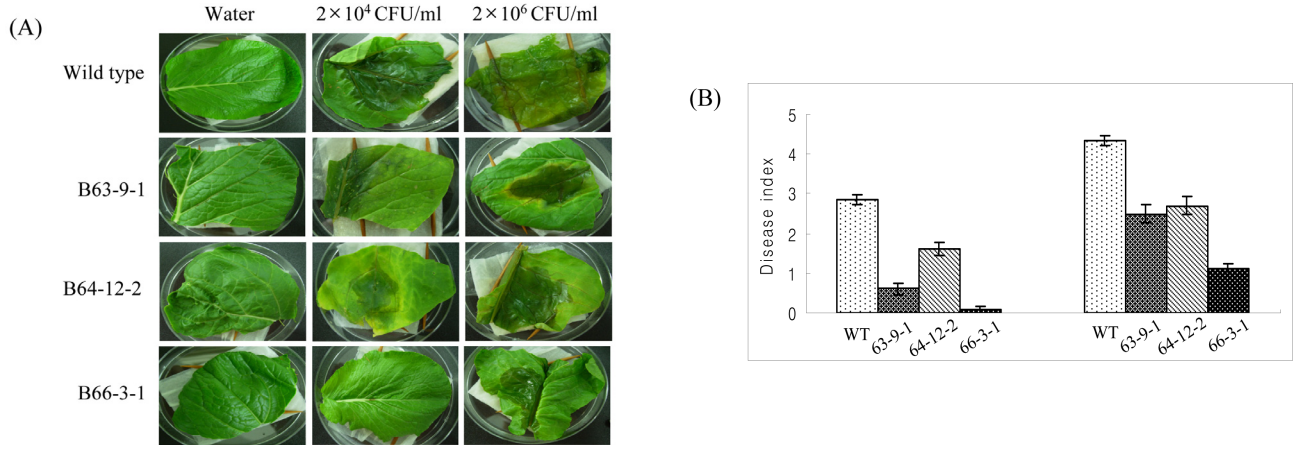

Fig. 3 Enhancing Bacterial Disease Resistance in Transgenic plants to 2days leaf after infection

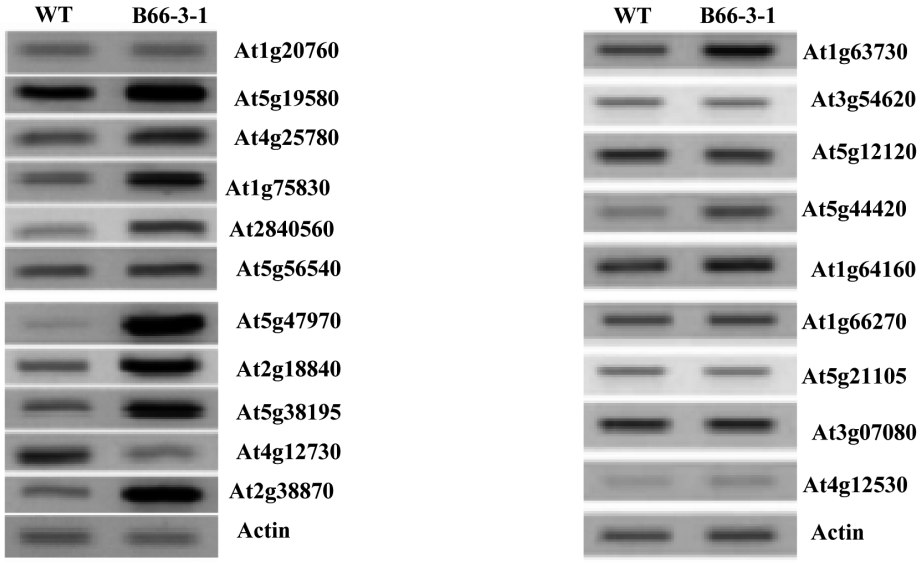

Fig. 4 Semi-quntitative RT-PCR analysis of expression levels of candidate genes identified by microarray analysis in B66-3-1 line and wild type control

tovorum, a disease index (DI) was calculated in 2×10^4 and 2×10^6 CFU two days after inoculation. Upon infection with 2×10^4 CFU, B63-9-1 and 64-12-2 showed DI of 0.8 and 1.4, respectively, compared with 2.9 DI of WT, while B66-3-1 did not show disease symptoms (Fig. 3A, B). Upon infection with 2 x 106 CFU, WT exhibited severe soft rot disease symptoms after two days, and died four days after infection, whereas transgenic lines showed much weaker symptoms as compared to WT (Fig. 3B).

Microarray analysis of transgenic line

In order to determine the global effects on BAA1 over-expression, mRNA transcription levels in leaves were compared between the WT and transgenic line (B66-3-1) by means of cDNA microarray analysis as described in Materials and Methods. Following data processing, several genes were shown to be consistently and significantly up- regulated, and others down-regulated in B66-3-1 as compared with WT. The filtering strategy selected changes on the basis of low *P* value (less than 0.05), combined with the fold change in expression, for signals that were significantly greater than the background noise. The results showed that, of the 2,013 genes analyzed, 23 genes (1.14% of total) were up-regulated and 11 genes down-regulated (0.55% of total) in the transgenics compared to the WT (Table 2). Interestingly, genes encoding defensin protein such as glyoxal oxidase, PR-1 protein, PDF1, protein kinase, LTP protein and UBA protein were up-regulated in the B66-3-1 line. Genes for cathepsin B-like protein, fatty acid desaturase family protein, tudor domain protein, thylakoidmembrane formation protein and helicase related protein were down- redulated in the B66-3-1 line.

Table 2 Significantly up-regulated genes in the BAA1 transgenic *B. rapa* over the wild type counterpart using Arabidopsis 37k oligo chip

Annotation	Gene ID	Expression levels		
		Transgenic (T)	Wild type (W)	Ratio (T/W)
Calcium-binding EF hand family protein	At1g20760	40627	3621	11.22
Putative protein	At4g33666	18144	1728	10.50
Glyoxal oxidase precusor from Phanerochaete chrysosporium	At5g19580	10020	1066	9.40
PR-1 protein from Medicago truncatula	At4g25780	35128	5816	6.04
Cystein-rich antifungal protein precursor (AFP1)	At1g75830	7432	1249	5.95
Protein kinase family protein	At2g40560	4638	876	5.35
Arabinogalactan-protein (AGP 14)	At5g56540	27891	5545	5.03
Nitrogen regulation family protein	At5g47970	17148	3672	4.67
Integral membrane Yip1 family protein	At2g18840	19970	4332	4.61
Protease inhibitor contains lipid transfer protein (LTP)	At5g38195	6781	1766	3.84
Fasciclin-like arabinogalactan-protein (FLA 2)	At4g12730	10052	2717	3.70
Protease inhibitor (BGIA) from Momordica charantia	At2g38870	29442	8412	3.50
Disease resistance protein (TIR-NBS-LRR class)	At1g63730	3406	973	3.50
BZIP transcription factor family protein	At3g54620	2142	619	3.46
Ubiquitin-associated (UBA)/TS-N domain protein	At5g12120	11635	3412	3.41
Unknown Protein	At5g06180	12975	3908	3.32
Plant defensin protein family	At5g44420	15566	4217	3.30
Disease resistance-responsive family protein	At1g64160	12899	4006	3.22
Beta-glycosidase (PSR 3. 2)	At1g66270	13121	4139	3.17
L-ascorbate oxidase from Nicotiana tabacum	At5g21105	5476	1755	3.12
Membrane protein contains Pfam	At3g07080	20826	6718	3.10
Protease inhibitor/seed storage/lipid transfer protein	At4g12530	7332	2365	3.10
DNA helicase (RECQ11)	At3g05740	5382	1794	3..00
Non-race specific disease resistance protein	At3g20610	9289	3248	2.86
O-methyltransase	At5g53810	20028	7102	2.82
Mitochondrial import receptor subunit TOM7	At5g41685	17702	6322	2.80
Auxin-responsive protein	At4g12980	11463	4230	2.71
Disease resistance-responsive protein	At2g21100	2612	997	2.62
Cellulose synthase	At5g44030	4167	1877	2.22

The results of the microarray experiment were confirmed by semi-quantitative RT-PCR for a number of the most significant changes identified (Fig. 4). The expression pattern of this set of genes was also determined in B66-3-1 and compared to WT. The expression levels of AFP1 encoding cystein-rich antifungal protein, GDH encoding glutamate dehydrogenase, PKC encoding protein kinase and LTP encoding protease inhibitor increased in the B66-3-1 line as compared to WT. Whereas the genes encoding the amino acid transporter protein and nicotianamine synthase were decreased in the B66-3-1 line as compared to WT.

Discussion

Cysteine proteases are found in bacteria, eukaryotic microorganisms, animals and plants (Barrett 1986). Many cysteine proteases have been identified in plants, where they participate in diverse metabolic events of physiological importance. The *BAA1* gene encoding cysteine protease has been cloned from pineapple fruit (Muta et al. 1997). However there are no reports on the function of *BAA1* gene. In the present study, we developed transgenic Chinese cabbage by introducing the *BAA1* gene isolated from pineapple fruit, and monitored its functional analysis using molecular and biological approaches. Several transgenic lines with normal horticultural traits and development were generated from this work, and most of them carried a single copy of the transgene. The *BAA1* transgenic Chinese cabbage lines showed variable levels of transgene expression at both the transcriptional and translational levels which did not correlate with transgene copy number. Our result provides the first evidence that it is possible to improve plant resistance by overexpression of gene encoding cysteine protease isolated from pineapple into Chinese cabbage. The 35S:BAA1 transgenic lines exhibited enhanced resistance to bacteria

soft rot infection. This resistance was manifested in the clearly reduced ability of the bacteria to establish infection, suggesting that the early events in this interaction have been altered; important since many studies about enhanced disease-resistance in transgenic cabbage plants have been reported (Hennin et al. 2001; Park et al. 2002; Cui et al. 2002; Wretblad et al. 2003). When infection and tissue maceration were initiated, however, the BAA1 protein appeared to be of less value because we could not observed any significant difference in bacterial growth between 35S:BAA1 transgenic and WT plants. However, when the soluble proteins (extra- and intra-cellular) of BAA1-overexpressed yeast was added to the growth medium, the growth of the *P. carotovorum ssp*. was significantly inhibited during the early growth phase. Therefore, we can conclude that the BAA1 protein produced by the recombinant yeast cell has antimicrobial activity against *P. carotovorum* especially in the dilute conditions of the cell. The results support our hypothesis about the role of cysteine protease in pathogenicity, in which *P. carotovorum* subsp. *carotovorum* employs quorum sensing to avoid the premature production of plant cell wall degradation enzymes (PCWDEs) (Andres et al. 2001). The results from microarray analysis revealed the up-regulation of defensin protein such as glyoxal oxidase, PR-1 protein, PDF1, protein kinase, LTP protein and UBA protein in the transgenic B66-3-1 line over the WT (Table 2). These results support the view that introduction of transgene of *A. comusus* to target plants are capable of protecting plants from bacterial pathogen infection through the production of antibacterial genes or hypersensitivity cell death (HCD) associated genes (Keller et al. 1999; Shen et al. 2000; Verberne et al. 2000). Also, overexpression of R2R3 MYB-related gene or AtMYB30 leads to the induction of HCD and could resist pathogen attack (Vailleau et al. 2000). Similarly Pti1, a serine/threonine kinase, overexpressed in tobacco accelerates the HCD and resists to infection of *P. syringae* pv. *tabaci* (Zhou et al. 1995). One of the most remarkable features in gene expression in our transgenic B66-3-1 line was the up-regulation of genes encoding arabinogalactan proteins (AGPs), fasciclin-like arabinogalactan proteins (FLAs). These AGP proteins regulate cell-cell interactions and communication, and provide key structural, positional and environmental signals during growth and development (Kim et al. 2003; Markus et al. 2006). Also the increased BAA1 expression could be an important factor responsible for the enhanced tolerance to multiple environmental stresses in our transgenic plants. WRKY transcription factors (Rizhsky et al. 2002; Kalde et al. 2003) and protein kinases (Saijo et al. 2000; Yoshida et al. 2002) have also been implicated in plant defense to environmental stresses. The possible contribution of up-regulation of genes encoding proteins associated with the enhanced stress tolerance in our transgenic Chinese cabbage requires further investigation.

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