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ORIGINAL PAPER

Enhancement of tolerance to soft rot disease in the transgenic Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) inbred line, Kenshin

Enkhchimeg Vanjildorj · Seo Young Song · Zhi Hong Yang · Jae Eul Choi · Yoo Sun Noh · Suhyoung Park · Woo Jin Lim · Kye Man Cho · Han Dae Yun · Yong Pyo Lim

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Abstract We developed a transgenic Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) inbred line, Kenshin, with high tolerance to soft rot disease. Tolerance was conferred by expression of *N*-acyl-homoserine lactonase (AHL-lactonase) in Chinese cabbage through an efficient *Agrobacterium*-mediated transformation method. To synthesize and express the AHL-lactonase in Chinese cabbage, the plant was transformed with the *aii* gene (AHL-lactonase gene from *Bacillus* sp. GH02) fused to the PinII signal peptide (protease inhibitor II from potato). Five transgenic lines were selected by growth on hygromycin-containing medium (3.7% transformation efficiency). Southern blot analysis showed that the transgene was stably integrated into the genome. Among these five transgenic lines, single copy number integrations were observed in four lines and a double copy number integration was observed in one transgenic line. Northern blot analysis confirmed that *pin*IISP-*aii* fusion gene was expressed in all the transgenic lines. Soft rot disease tolerance was evaluated at tissue and seedling stage. Transgenic plants showed a significantly enhanced tolerance (2–3-fold) to soft rot disease compared to wild-type plants. Thus, expression of the fusion gene *pin*IISP-*aii* reduces susceptibility to soft rot disease in Chinese cabbage. We conclude that the recombinant AHL-lactonase, encoded by *aii*, can effectively quench bacterial quorum-sensing and prevent bacterial population density-dependent infections. To the best of our knowledge, the present study is the first to demonstrate the transformation of Chinese cabbage inbred line Kenshin, and the first to describe the effect of the fusion gene *pin*IISP-*aii* on enhancement of soft rot disease tolerance.

Keywords *Brassica rapa* L. ssp. *pekinensis* inbred line · Kenshin · *Agrobacterium*-mediated transformation · Soft rot disease tolerance · Autoinducer inactivation · Potato proteinase inhibitor II · *pin*IISP-*aii* fusion gene

Abbreviations

2,4-D	2,4-Dichlorophenoxy-acetic acid
BA	N^6-Benzyladenine
NAA	α-Naphthalene acetic acid
aii	Autoinducer inactivation
PinII	Potato proteinase inhibitor II
SP	Signal peptide
GFP	Green fluorescent protein
HPTII	Hygromycin phosphotransferase II

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E. Vanjildorj · S. Y. Song · Z. H. Yang · Y. P. Lim (✉)
Department of Horticulture, Chungnam National University, Gung-dong, Yuseong-gu, Daejeon 305-764, Korea
e-mail: yplim@cnu.ac.kr

J. E. Choi
Division of Plant Science and Resources, Chungnam National University, Daejeon 305-764, Korea

Y. S. Noh
Department of Biological Sciences, Seoul National University, Seoul 151-742, Korea

S. Park
Vegetable Division, NHRI, RDA, Suwon 441-440, Korea

W. J. Lim · H. D. Yun
Division of Applied Life Science (BK21 Program), Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, Korea

K. M. Cho
Department of Food Science, Jinju National University, Chinju 660-758, Korea

AHL *N*-acyl homoserine lactone
CFU Colony-forming unit

Introduction

Brassica rapa L. ssp. *pekinensis* (Chinese cabbage) is one of the most important vegetables in Korea, as emphasized by extensive agriculture across 37,200 ha. Chinese cabbage is usually used as the base ingredient of kimchi, the most favorable and common side dish eaten at every Korean meal with rice (Park and Cheigh 2003). A major advantage of consuming Chinese cabbage is the prevention of colon cancer by secondary metabolites, e.g. glucosinolates. Chinese cabbage is a good source of carbohydrates, vitamins A and C, folic acid, and minerals, including calcium, potassium, and iron (Bender and Bender 1995).

The Chinese cabbage inbred line, Kenshin, is one of the parental lines of a CKDH mapping population that was used to construct a reference genetic map for *Brassica rapa* genome sequencing. To maximize the utility of Kenshin as a reference genetic material for functional genomic research, it is necessary to develop a simple and efficient *Agrobacterium*-mediated transformation system.

Bacterial soft rot is the most severe and destructive disease across members of *Brassica*. Chinese cabbage is highly susceptible to bacterial soft rot disease, which is caused by a Gram-negative bacterium, *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). In general, *B. rapa* has the highest soft rot disease severity rating compared to other susceptible species, and the most susceptible *B. rapa* subspecies are *pekinensis* and *chinensis* (Ren et al. 2001b). Control of soft rot disease is difficult due to a wide range of hosts, the ability of the bacteria to survive in the plant debris in soil, and host susceptibility. Chemical controls are not available. Culturing practices, including raised planting beds, reduced plant density, and delayed planting dates can reduce disease frequency and progression, but may reduce agricultural efficiency (Fritz and Honma 1987). Therefore, genetic tolerance or resistance may represent an ideal alternative approach. Ren et al. (2001a) improved soft rot disease resistance of Chinese cabbages through repeated intercrossing. But the traditional recurrent selection procedure is time- and labor-consuming. Moreover, due to lack of natural resistant gene against soft rot disease, the proceeding of routine raising disease-resistant breeding is restricted (Li 1995). Therefore, genetic engineering of soft rot disease tolerance in Chinese cabbage is of significant interest to agricultural biotechnology. Chinese cabbage is generally considered to be recalcitrant in tissue culture (Zhang et al. 1998), and there are only few reports on the transformation of hybrid lines (Jun et al. 1995; Zhang et al. 2000; Cho et al. 2001; Zhao et al. 2006; Kim et al. 2007) and some inbred lines (Kim et al. 2003; Min et al. 2007). None of these studies involved the transfer of soft rot disease tolerant or resistant genes. But recently, transgenic Chinese cabbage plants overexpressing *BAA1* gene was reported to show resistance to bacterial soft rot disease caused by *Pcc* (Jung et al. 2008).

In Gram-negative bacteria, including *Pcc* that causes soft rot disease in plants, autoinducers such as *N*-acyl homoserine lactones (AHLs) regulate particular virulence gene expression in plants and animals. The autoinducer inactivation gene (*aii*) family members encode AHL inactivation enzymes (e.g. AHL-lactonase) that significantly reduce the release of AHLs, decrease extracellular pectolytic enzyme activities and attenuate pathogenicity of *Pcc* for potato, eggplant, Chinese cabbage, carrot, celery, and tobacco (Dong et al. 2000, 2001).

Proteins transport through the plasma membrane to the outside of the cell in a manner that requires a signal peptide, such as that of potato proteinase inhibitor II (PinII). Signal peptides may target the recombinant proteins to the apoplast of transgenic tobacco and potato plants (Herbers et al. 1995; Murray et al. 2002; Farran et al. 2002; Liu et al. 2004). Therefore, the *pin*IISP-*aii* fusion gene may target AHL-lactonase to the intercellular space of Chinese cabbage tissues where *Pcc* initiates infection. The objective of present study was to introduce the fusion gene (*pin*IISP-*aii*) into Chinese cabbage inbred line Kenshin genome to produce genetically modified plants capable of quenching pathogen quorum-sensing signaling.

Materials and methods

Preparation of cotyledon explants

Seeds of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*, inbred line Kenshin) were surface sterilized with 70% ethanol for 1 min, followed by sodium hypochlorite (2% active chloride) for 20 min. After thorough washing with sterile distilled water the seeds were plated on MS (Murashige and Skoog 1962) medium containing 30 g l^{-1} sucrose and 8 g l^{-1} phytoagar. Cultures were maintained at 25 ± 1°C with 16/8 h day/night photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamp. After 4 days of culture, cotyledons were excised and used for further experiments. All cultures, except those for co-cultivation, were maintained under the same in vitro conditions described above.

Bacterial strains, plasmids, and media

Bacillus sp. GH02 was isolated from ginseng root (Cho et al. 2007) and was cultured at 28°C. The pGEM-T Easy

vector (Promega, WI, USA) was used for cloning and sequencing.

The transformation of Chinese cabbage was performed with a binary vector pCAMBIA1302 (CAMBIA, Australia) which contains the fusion gene (*pin*IISP-*aii*), signal peptide of protease inhibitor II from potato (PinII) fused to autoinducer inactivation (*aii*) gene (GenBank accession number, FJ189472) from *Bacillus* sp. GH02, the green fluorescent protein (*gfp*) gene, and a hygromycin phosphotransferase II (*hpt*II) gene for resistance to hygromycin (Fig. 2a). The binary vector was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation (Gene Pulser II Electroporator, Bio-Rad, Hercules, CA, USA). *A. tumefaciens* strains were grown overnight in YEP medium prior to use in transformation.

Recombinant DNA techniques

Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gel, DNA ligation, and other cloning-related techniques were followed (Sambrook and Russell 2001). Nucleotide sequences were determined using the dideoxy chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT, USA). Assemblages of the nucleotide sequences and amino acid sequence analysis were done using the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). DNA and amino acid sequence homology searches were performed using the National Center for Biotechnology Information (NCBI) website with the BLAST network service and the nonredundant DNA and protein sequence databases.

Cloning of *aii* genes

To amplify autoinducer inactivation (*aii*) gene homologs from the quorum-quenching bacterial chromosome (*Bacillus* sp. GH02), degenerate oligonucleotide primers were designed based on conserved amino acid sequences from highly conserved regions. The sense and antisense degenerate oligonucleotide primers used were 5′-CAC YTR CAT YTT GAY CAY GC-3′ (sense, 694F) and 5′-ATC RAA TCC HGA YRA YGG C-3′ (antisense, 695R), respectively. PCR amplification using genomic DNA of *Bacillus* sp. GH02 as a template was carried out using Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK) under 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. The amplified product of approximately 300 bp was isolated from an agarose gel using a gel extraction kit (Intron Biotechnology, Seongnam, Korea). The PCR product was sequenced, the sequence was confirmed by BLAST searches. From the sequenced DNA the 5′ upstream and 3′ downstream regions were amplified by primer walking using only gene specific primers based on the determined sequence. The amplified fragments were isolated for further nucleotide sequencing. The complete open reading frame (ORF) was amplified from genomic DNA using specific primers (705F and 803R) and cloned into the pGEM-T easy vector (Table 1). Reported nucleotide sequence data is available in the GenBank database under the accession number FJ189472 for the *aii* gene, and DQ365557 for the 16S rDNA of *Bacillus* sp. GH02.

Construction of the *pin*IISP-*aii* fusion gene

For the construction of the fusion gene *pin*IISP–*aii*, the potato *pin*IISP gene and *Bacillus* sp. GH02 *aii* gene were amplified from chromosomal DNA with the primer set, *pin*IISP (1948F–1950R) and *aii* (1949F–1099R), respectively (Table 1). Overlap sites were introduced into 1949F–1950R. To express fusion genes, the *pin*IISP and *aii* gene fragments were amplified with primers 1948F–1950R and 1949F–1099R, then purified by gel extraction using a kit (Intron Biotechnology, Seongnam, Korea), ligated into pGEM-T easy vector and were transformed into *E. coli* DH5α.

Agrobacterium-mediated transformation procedure

Five microliters of *A. tumefaciens* stock was cultured in 5 ml YEP medium containing 100 mg l^{-1} kanamycin at 28°C. After 24 h of culture, 10 μl of aliquot was sub-cultured again in 10 ml YEP medium with 100 mg l^{-1} kanamycin until an OD_{600} value of 0.6. The bacterial suspension was centrifuged at 3,500 rpm for 15 min, and

Table 1 List of oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′ → 3′)
694F	CAC YTR CAT YTT GAY CAY GC
695R	ATC RAA TCC HGA YRA YGG C
705F	TCA AAA CTA AAT GTA AAG GTG G
803R	CAT ATG GGT AGT TGA YCA TC
1098F	AAA ATC TAG ACA AAA CTA AAT GTA AAG GTG *Xba*I
1099R	TTT CTC GAG TAT ACC CAT CAA CTR GTA G *Sac*I
1948F	AAA TCT AGA ACA GAC ACT CTT CAC CCC AA *Xba*I
1949F	AGC GCG ATG GAG CAT TTA GAC TTA CCG GTT
1950R	AAC CGG TAA GTC TAA ATG CTC CAT CGC GCT

Underlined is the indicated restriction enzyme site such as *Xba*I (forward) and *Sac*I (reverse)

the pellet was resuspended in 10 ml of resuspension medium (MS with 50 mg l^{-1} acetosyringone). The cotyledonary explants (0.3 × 0.3 cm^2) were cultured on pre-cultivation medium—MSRM (MS with 5 mg l^{-1} BA, 0.5 mg l^{-1} NAA, 2 mg l^{-1} $AgNO_3$, 16 g l^{-1} phytoagar, pH 5.8) in the light for 3 days. For co-cultivation, the pre-cultured explants were immersed in bacterial suspension for 10 min and then cultured on co-cultivation medium (MSRM with 50 mg l^{-1} acetosyringone) in the dark at 25°C for 3 days. Thereafter, the explants were rinsed three times in sterile distilled water, and once in 500 mg l^{-1} carbenicillin containing-sterile distilled water for 10 min, and then surface-dried on sterilized filter paper. For selection, the explants were transferred to callus regeneration/selection medium (MSRM with 250 mg l^{-1} carbenicillin and 10 mg l^{-1} hygromycin). After 5 weeks of selection, all the hygromycin-resistant calli were transferred to shoot regeneration/selection medium (MS with 2 mg l^{-1} BA, 0.5 mg l^{-1} NAA, 5 mg l^{-1} $AgNO_3$, 250 mg l^{-1} carbenicillin, 10 mg l^{-1} hygromycin, 8 g l^{-1} phytoagar, pH 5.8). After 6 weeks of culture, hygromycin-resistant multiple shoots were transferred to the medium (MS with 250 mg l^{-1} carbenicillin, 10 mg l^{-1} hygromycin, 8 g l^{-1} phytoagar) to produce the adventitious shoots. After 2 weeks of culture, the adventitious shoots were transferred to rooting medium (1/2 MS with 250 mg l^{-1} carbenicillin, 10 mg l^{-1} hygromycin, 8 g l^{-1} phytoagar). After 3 weeks of culture, the rooted plants were transferred to the soil. Two weeks later, the hygromycin-resistant plants were transferred to 4°C and cultured during 45 days for vernalization. Finally, the plants were transferred to greenhouse and then self-pollinated.

Transgene segregation test in T_1 progenies

Self-pollinated seeds of wild-type and T_0 transgenic plants were cultured on selection medium (MS with 25 mg l^{-1} hygromycin, 8 g l^{-1} phytoagar). The treatment was repeated three times in each transgenic line. After 5 weeks of culture, hygromycin-resistant plants developing true leaves were counted, and data were analyzed using the χ^2 test at $P = 0.05$.

Southern and Northern blot analyses

Genomic DNA was extracted from young leaf tissues using Cetyl trimethylammonium bromide (CTAB) (Saghai-Maroof et al. 1984). About 30 μg of *Hin*dIII-digested genomic DNA samples were electrophoresed on an 0.8% (w/v) agarose gel, then depurinated in 0.25 N HCl for 10 min, denatured in 0.5 N NaOH and 1.5 N NaCl for 1 h, neutralized in 1 M Tris (pH 7.5) and 1.5 M NaCl for 1 h, and washed in 10 × SSC for 1 h.

For studying transgene expression, total RNA was extracted from young leaf tissues using Tri-Reagent (MRC Inc., USA). About 30 μg of RNA from each sample was electrophoresed on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde. Gels were blotted onto a positively charged nylon membrane (Amersham Pharmacia Biotech, UK) using 20 × SSC by download capillary transfer method, and probed with a ^{32}P labeled *pin*IISP-*aii* gene probe, which was a PCR-amplified product. *pin*IISP-*aii* gene was amplified using primer set, 5′- TAC TTG TAA GCG CGA TGG AG -3′ (forward) and 5′- AGA TGA AGT GCC ATT TGC G -3′ (reverse), yielded about 532-bp PCR product. The following PCR conditions were used: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s (33 cycles). Hybridization was performed overnight at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech, UK). Subsequent steps of Southern and Northern blot analyses were performed as described by Enkhchimeg et al. (2005).

Tissue inoculation

The *Pcc*, used in the present study, was isolated from Chinese cabbage tissue with soft rot symptoms. The *Pcc* stock (10 μl) was cultured in 25 ml of liquid YEP medium until $OD_{600} = 1.4$ equals 1,170,000 CFU ml^{-1} and then diluted it to $OD_{600} = 0.5$. Second leaves from 2-month-old wild-type and T_1 transgenic plant lines T101 and T103, the progenies of T1 and T3, were excised and then placed in Petri dishes that contained teeth sticks on two layers of Whatman filter paper moistened with 7 ml of sterile water. For inoculation of plants, only YEP or 100 μl YEP medium containing actively growing bacteria ($OD_{600} = 0.5$ equals 420,000 CFU ml^{-1}) was added to the freshly needle-wounded site of the midrib of the detached leaves, and incubated at 28°C for 6 days. All inoculations were performed three times, and the pathogenicity was determined by disease lesion diameter after 1, 3, and 6 days of the inoculations, respectively. The data analysis was done with SAS 9.1 (SAS Institute Inc., Cary, NC USA) and was compared by ANOVA (one way) with Duncan's multiple range test ($P > 0.05$).

To determine the minimum lethal density of bacteria, and optimal bacterial activity for wild-type plants, different population densities ($OD_{600} = 0.25$, 0.5, 0.7, 1.0, 1.2, and 1.4) were tested. Inoculation was carried out as described above.

Plant inoculation

Self-pollinated seeds of wild-type and T_0 transgenic plants of T1 and T3 transgenic lines were cultured on the plant segregation medium (MS with 25 mg l^{-1} hygromycin, 8 g l^{-1} phytoagar). After 4 weeks of the culture, T_1

hygromycin-resistant plants of T101 and T103 transgenic lines were transplanted into plastic pods with peat moss and perlite (1:1). The plants were covered by wrap with small holes and grown in the culture room adjusted to 25 ± 1°C and 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. Approximately in 10 days of transplantation, the plants at the 5–7-leaf stage were used to evaluate of tolerance against soft rot disease.

For plant inoculation, *Pcc* stock (10 μl) was cultured in 25 ml of liquid YEP medium until $OD_{600} = 1.4$ equals 1,170,000 CFU ml^{-1} and then diluted it to $OD_{600} = 0.1$. The plant inoculation was performed as described by Lee and Cha (2001). Each plant was drenched by pouring 5 ml of a 4:1 mixture of *Pcc* and sterile mineral oil (heavy white oil, Sigma) over the center of the plant. The inoculated plants were then covered by wrap with small holes and grown in the culture room as described above. The soil, peat moss and perlite (1:1), was kept with constantly enough moisture. The inoculated plants were examined for soft rot daily after *Pcc* inoculation. The disease test was performed on 25 plants per replicate, and it was repeated three times. The data analysis was done with SAS 9.1 (SAS Institute Inc., Cary, NC USA), and was compared by ANOVA (one way) with Duncan's multiple range test ($P > 0.05$).

Results and discussion

Construction of the fusion gene by overlap PCR

To isolate the *aii* gene from the strain GH02, primers designed from the conserved regions found in several groups of metallohydrolases, including AHL-lactonases from *Bacillus cereus* group (Dong et al. 2000; Lee et al. 2002) and *A. tumefaciens* (Zhang et al. 2002), were used. The *aii* of *Bacillus* sp. GH02 is 753-bp long and encodes a protein of 250 amino acids with a predicated molecular mass of 28,056 Da. Comparison of the *Bacillus* sp. GH02 and *Bacillus* sp. 240B1 *aii*A (Dong et al. 2002) deduced amino acid sequences revealed 93% amino acid sequence identity. The *pin*IISP and *aii* genes were fused by overlap PCR. The 1,103 bp (*pin*IISP-*aii*) DNA fragments were ligated into the pGEM-T Easy vector and transformed into *E. coli* DH5α (data not shown).

Regeneration of hygromycin-resistant plants

To develop the soft rot disease tolerant Chinese cabbage inbred line, Kenshin, 4-day-old cotyledon explants were inoculated with *A. tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA1302 carrying *pin*IISP-*aii*, *gfp*, and *hptII* genes. A co-cultivation period of 2 or 3 days was determined to be optimal. Usually, explants of *Brassica* are very sensitive to co-cultivation with *Agrobacterium*, and a co-culture extension period caused necrosis during subsequent cultivation.

Within 5 weeks of selection, eight (of 215 total) hygromycin-resistant calli were regenerated from the cut ends of the explants, resulting in 3.7% transformation efficiency (Fig. 1a). These calli did not regenerate any shoots when continuously subcultured on the fresh medium containing 16 g l^{-1} phytoagar; however, 16 g l^{-1} of phytoagar was found to be the best for shoot regeneration from inbred lines of Chinese cabbage (Yang et al. 2004). This phenomenon is consistent with a previous report (Zhang et al. 2000), and suggests that the callus on the cut edges of the explants proliferated more on higher concentrations of phytoagar-containing medium. Even though *B. rapa* is the most recalcitrant species compared to other *Brassica* species, Zhao et al. (2006) successfully generated transgenic Chinese cabbage by applying a high concentration of silver nitrate in regeneration medium. Therefore, we modified the shoot regeneration/selection medium such that it contained a high concentration (5 mg l^{-1}) of silver nitrate, as well as only 8 g l^{-1} of phytoagar (see in material and method) to promote shoot induction. After 6 weeks of culture, five out of eight independent lines of the calli generated multiple hygromycin resistant shoots (Fig. 1b). Adventitious shoots were generated on hygromycin-containing, hormone-free MS medium within 14 days of culture, and at the same time growth of multiple shoots was completely stopped (Fig. 1c). The surrounded multiple shoots and calli were then completely removed and only the adventitious shoots were cultured on hygromycin-containing 1/2 MS medium for rooting. Roots were successfully generated within 3 weeks of culture (Fig. 1d). Selection medium and rooting medium were refreshed once a week, which significantly promoted growth. Our *pin*IISP-*aii* transgenic plants exhibited neither growth inhibition nor visible phenotypic alternations (Fig. 1e). Generation of in vitro transgenic plants from seed culture took about 4 months.

In general, most *Brassica* species are vernalization-sensitive plants. Therefore, plants with 5–6 true leaves were vernalized for 45 days; during this period plant growth was almost completely inhibited (Fig. 1f). Then, plants were transferred to the greenhouse, and flower buds and male fertile flowers appeared after 10–15 days (Fig. 1g). Self-pollinated T_1 seeds were harvested after 2 months (Fig. 1h, i). While transgenic plants were phenotypically normal during tissue culture, one transgenic line (T5) exhibited male sterility. This could be due to somaclonal variation during tissue culture (Min et al. 2007).

Fig. 1 Regeneration of *pin*IISP-*aii* transgenic Chinese cabbage inbred line Kenshin via *Agrobacterium*-mediated transformation. **a** Hygromycin-resistant calli derived on the edges of cotyledon explants after 5 weeks of selection, **b** hygromycin-resistant multiple shoots regenerated after 6 weeks of culture, **c** hygromycin-resistant adventitious shoots regenerated within 14 days, **d** root regenerated within 3 weeks, **e** transgenic plants growing in the greenhouse, **f** vernalization at 4°C for 45 days, **g** transgenic plants at the flowering stage, **h** self pollination, **i** transgenic seed ripening, **j** transgene segregation in T_1 progenies. Self-pollinated seeds of wild-type (*left*) and T_1 transgenic plants (*right*) were germinated on 25 mg l^{-1} hygromycin containing MS medium

Transgene segregation in T_1 progeny of transgenic plants was determined on 25 mg l^{-1} hygromycin containing medium. All four transgenic lines showed 3:1 segregation ratios ($\chi^2 = 0.003$–0.04, $P = 0.84$–0.96), suggesting single copy integration of the transgenes in the T_1 progenies of transgenic plants (Table 2; Fig. 1j).

*pin*IISP-*aii* transgene integration and expression

Integration of the *pin*IISP-*aii* transgene into the genome of transgenic plants was confirmed by Southern blot analysis. A single copy of the transgene was successfully integrated into each of the four transgenic lines (T1–T4), and two copy numbers transgene were integrated in one transgenic line (T5) but not in wild-type plant (Fig. 2b). The expression of the *pin*IISP-*aii* transgene in the transgenic plants was confirmed by Northern blot analysis. The *pin*IISP-*aii* transcript was detected in all the transgenic plants but not in the wild-type plant (Fig. 2c).

The concept of plant transformation combines components of both plant regeneration and *Agrobacterium* related parameters, such as virulence induction, T-DNA activation, transfer, and integration. With all these factors interacting during the induction of transgenic plants, establishing optimal conditions can be extremely difficult. Additionally, regeneration and transformation in *Brassica* is highly genotype-dependent (Cardoza and Stewart 2004). Variation was observed in regeneration frequency across the 123 genotypes of Chinese cabbage tested, ranging from 95 to 0% (Zhang et al. 1998). Thus, genotype specificity is a limiting factor in *Brassica* regeneration, and in further transformation. Therefore, there is still a need for developing simple and efficient transformation methods to overcome *Brassica* genotype dependency. The best transformation method must be that it is applicable to the wide range of genotypes of Chinese cabbage.

The transformation method we present has been tested with three genes (*pin*IISP-*aii*, *CHITINASE*, and pathogenicity quenching factor3-*Pqf3*) with transformation efficiencies of 3.7, 4.3, and 3.0% into genome of inbred line Kenshin. This method was also used with the *Pqf3* gene, where transformation efficiencies of 1.4 and 10.5% were

Table 2 Segregation of hygromycin resistance in T_1 progeny of *pin*IISP-*aii* transgenic plants

Transgenic lines	Resistant (R) progeny	Susceptible (S) progeny	Ratio (R:S)	Expected ratio	χ^2 value	*P* value
T101	8.7 ± 3.0	3.3 ± 1.1	2.6:1	3:1	0.04	0.84
T102	12.5 ± 2.1	4.3 ± 1.0	2.9:1	3:1	0.003	0.96
T103	11.8 ± 1.7	4.3 ± 1.4	2.7:1	3:1	0.02	0.89
T104	6.7 ± 0.9	2.1 ± 0.5	3.2:1	3:1	0.007	0.93

Each value represents mean ± SD of three replicates. χ^2 indicates the significant fit to the expected ratio

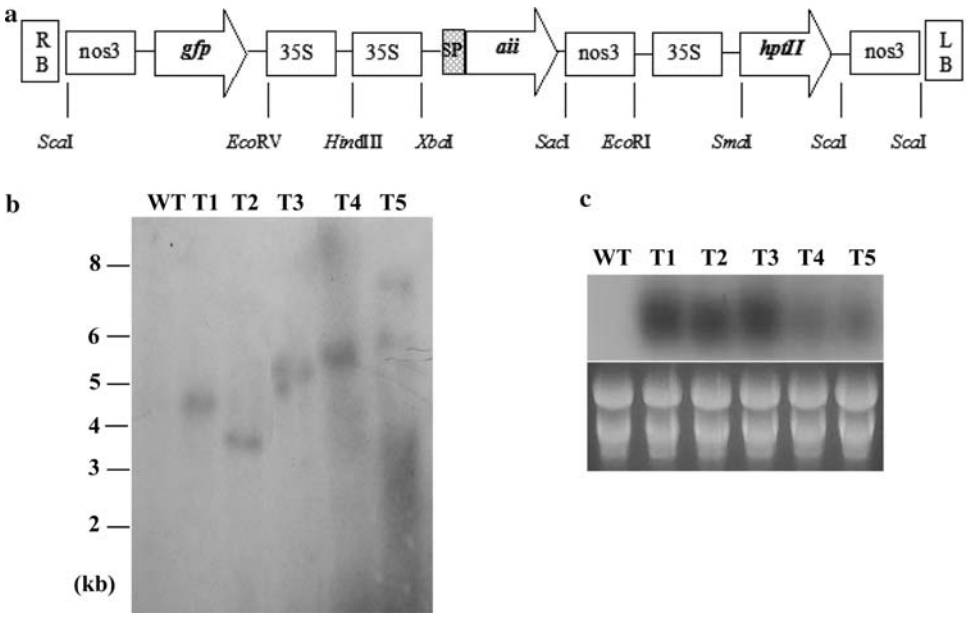

Fig. 2 **a** Schematic diagram of the T-DNA region of the binary vector pCAMBIA1302. *gfp* green fluorescent protein gene; *SP* protease inhibitor II signal peptide from potato; *aii* autoinducer inactivation gene from *Bacillus* sp. GH02; *hptII* hygromycin phosphotransferase II gene; *CaMV 35S* cauliflower mosaic virus 35S promoter; *nos3* nopaline synthase transcription terminator; *RB and LB* right and left borders. **b** Southern blot analysis of wild-type (WT) and T_0 *pin*IISP-*aii* transgenic lines T1–T5. About 30 μg of *Hin*dIII-digested genomic DNA from each sample was probed with a ^{32}P labeled *pin*IISP-*aii* probe. **c** Northern blot analysis of mRNAs from wild-type (WT) and the transgenic lines T1–T5. About 30 μg of total RNA per sample was probed with the above probe. The *bottom panel* shows EtBr staining of the RNA gel

observed for into genomes of inbred line Chiifu and the hybrid line C × K (Chiifu × Kenshin), respectively, which are the other primary strains used in the multinational *Brassica rapa* genome sequencing project (data not shown). When directly compared between inbred lines, Kenshin exhibited 2–3-fold higher transformation efficiencies than Chiifu. This presents Chinese cabbage inbred line Kenshin as a more regeneratable and reproducible in tissue culture and transformation than Chiifu. Furthermore, the hybrid line (C × K) exhibited 2–8-fold higher transformation efficiency than these inbred lines. This data collectively suggests that the transformation of inbred lines of Chinese cabbage is much more difficult than hybrid lines. This variation in transformation efficiency was dependent on genetic background differences of Chinese cabbage cultivars.

In previous studies, several genes were successfully transformed into Chinese cabbage hybrid lines, including a TMV coat protein gene with 4% of transformation efficiency (Jun et al. 1995), a binary vector pIG121Hm containing an intron-*gus* gene with 1.6–2.7% of transformation efficiencies (Zhang et al. 2000), a *cry1C* Bt gene with 0.4–8.9% of transformation efficiency (Cho et al. 2001), and an antibacterial peptide and *CpTI* genes with 2–17% of transformation efficiencies (Zhao et al. 2006). To our knowledge, only two studies have reported a successful gene transformation into Chinese cabbage inbred lines: Kim et al. (2003) transformed the *PDI* gene into cotyledon explants with 0.4–0.8% transformation efficiencies, and Min et al. (2007) used mannose selection to transform the *GLOase* and *JMT* genes to hypocotyls explants of inbred lines of Chinese cabbage with 1.4% and 3.0%

transformation efficiency. Even though transformation of Chinese cabbage, especially its inbred lines, has proved difficult for transgenic development, the Chinese cabbage inbred line, Kenshin, acquires the *pin*IISP-*aii* fusion gene with the 3.7% transformation efficiency. Although we used antibiotic selection, the final transformation efficiency was comparable to or better than that obtained from the previous studies.

In conclusion, we have developed a simple and efficient transformation system for the production of transgenic Chinese cabbage by *A. tumefaciens*-mediated transformation. The whole system is applicable in transferring other transgenes into both inbred and hybrid lines of Chinese cabbage, which could benefit both basic and applied research.

Evaluation of tolerance against soft rot disease

We found that the occurrence of typical maceration symptoms of soft rot disease was dependent on two major factors: pathogen density and bacterial activity. *Pcc* does not produce plant tissue-degrading enzymes until sufficient bacterial density is achieved for successful evasion of plant defenses. In the previous study, Dong et al. (2001) also reported that resistance in the *aiiA* transgenic plants is related to inoculated pathogen population density.

Due to long experimental culturing of bacteria, reduced activity with respect to infectivity might occur. Therefore, prior to plant inoculation in our experimental setup, the minimum fatal density of pathogen and optimal bacterial activity at an occurrence of typical maceration symptoms of soft rot disease on wild-type plants were determined. All pathogen population densities used in this study resulted in typical maceration symptoms on wild-type plants several hours after inoculation, although data at 6-day-after-inoculation indicated that the applied population density of pathogen at $OD_{600} = 0.5$, equaling 420,000 CFU ml^{-1}, caused the severest maceration symptom on wild-type plants compared to lower or higher pathogen densities (data not shown). Therefore, we applied the 420,000 CFU ml^{-1} pathogen density to the experimental transgenic plants in order to test the tolerance to soft rot disease.

Two T_1 transgenic lines, T101 and T103 showing high expression of *pin*IISP-*aii* gene (Fig. 2c), were selected for further experiment. Initially, disease symptom evaluation was carried out on tissues of detached leaves of wild type, T101 and T103 plants. Significant differences of disease symptom development were observed between wild type plants and transgenic lines at 1-, 3-, 6-, and 10-day post-inoculations, while two transgenic lines showed significant difference of disease development between them at 6- and 10-day post-inoculations (Fig. 3a). Greater symptoms of disease development in detached leaves of wild type plants were observed from 1-day post-inoculation to 10-day post-inoculation compared to transgenic lines, thereby suggesting that transgenic plants expressing *pin*IISP-*aii* experienced delayed soft rot symptom development. At 1-day post-inoculation, significantly greater maceration symptoms (0.3 cm^2 in size) were observed in wild-type plants compared to symptoms exhibited by transgenic plants, and no significant differences between individual transgenic lines were observed (Fig. 3a). After 3-day post-inoculation, disease symptoms of wild-type plants increased rapidly (3–4-fold), compared to transgenic plants, and maceration area of leave became watery-gray in wild-type plants and brown in transgenic plants (Fig. 3a, b). At 6-day post-inoculation, the disease symptoms reached up to 10.9 cm^2 in wild-type, 1.3 cm^2 in T101, and 4.2 cm^2 in T103. Maceration area of wild-type plants completely decayed but the area of transgenic plants dried and color turned dark brown (Fig. 3a, c). At 10-day post-inoculation, the disease symptoms of wild-type plants were distributed across the whole midrib of leaf tissues, and the tissues decayed and collapsed, whereas maceration stopped and black coloration observed in transgenic plants (Fig. 3a). The intensity of black staining can serve as a reliable qualitative marker for Chinese cabbage tissue infection by *Pcc*. Thus, transgenic plants expressing *pin*IISP-*aii* exhibited a significant increase in tolerance compared to wild-type plants against soft rot disease. Further observation of disease development for longer period could not be done in excise leave tissues because of discoloration that might be due to physiological stress experienced by detached leave tissues. Therefore, further evaluation of disease symptom development was carried out on whole plants at the seedling stage for longer period.

Induction of soft rot disease by artificial inoculation is important for many areas, such as breeding of resistance cultivars, evaluation of pesticides, and study of host–pathogen interaction. However, it is difficult to get uniform soft rot disease by inoculation. According to Lee and Cha (2001), an artificial inoculation was carried out by pouring the mixture of mineral oil and bacterial suspension on center of the plants without wounding and keeping them inside the moist chamber, whereas none of the plants showed soft rot symptom by just bacterial suspension.

In total of 44% of wild-type plants developed a typical disease symptom at 3-day post-inoculation, whereas 21.4% of T101 and 15.8% of T103 transgenic plants, respectively, developed the symptom at this time (Fig. 4a). At 7-day post-inoculation, percentage of wild-type plants with the disease symptom was reached to 52% (44% severe, 8% medium levels), while it was reached to 35.7% (21.4% severe, 14.3% medium levels) in T101 but not increased in T103 (Fig. 4a). At 14-day post-inoculation, 68% of

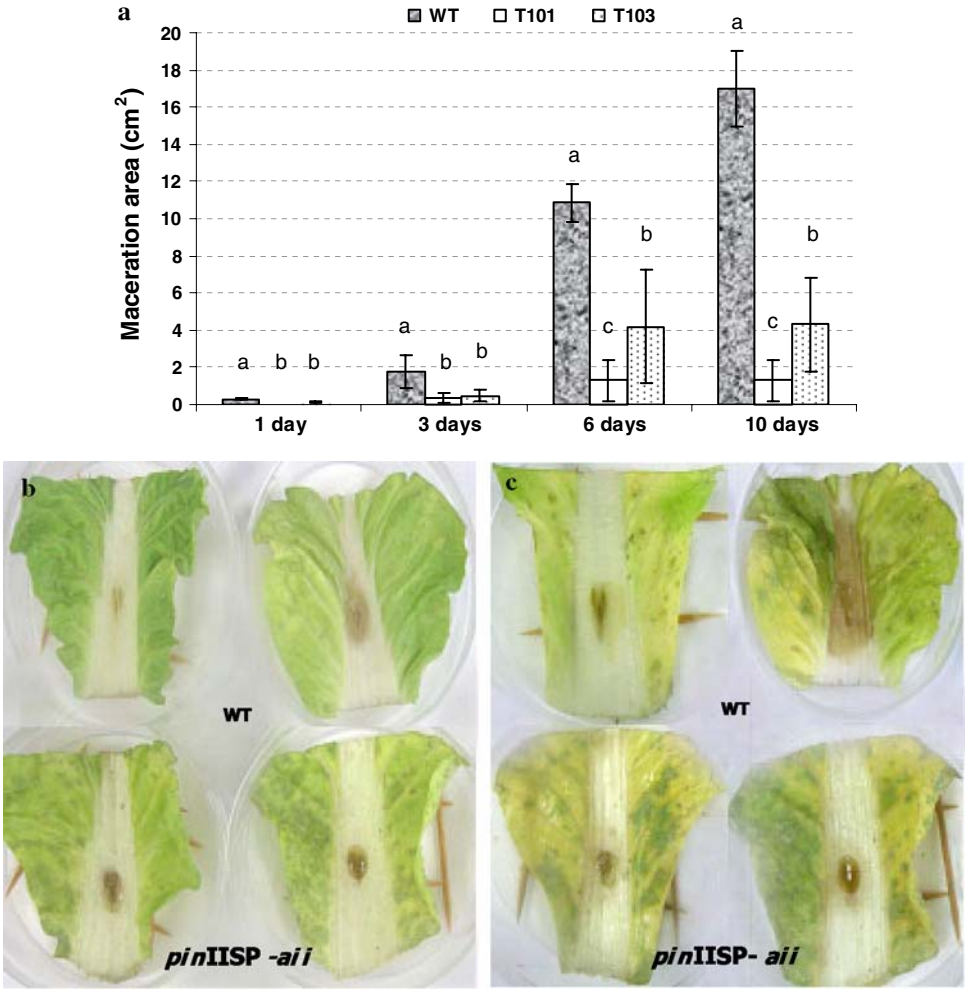

Fig. 3 Tissue inoculation with *Pectobacterium carotovorum* subsp. *carotovorum*. **a** Maceration area of wild-type (WT) and transgenic lines of T101 and T103 at 1-, 3-, 6-, and 10-day post-inoculation. *Gray* (WT), *white* (T101), and *dotted* (T103) columns indicate the mean of three replicates, and *bars* represent ±SD, respectively. Means indicated with *different letters* (*a*, *b*, and *c* on *top* of the *columns*) were significantly different between each other (ANOVA, Duncan's multiple range test, $P > 0.05$). **b**, **c** The freshly needle-wounded site of the midrib of leaf tissues from WT inoculated by only YEP medium (*top row, left*), and both WT and T_1 transgenic plant lines, T101 and T103, were inoculated with actively growing bacteria (*top row*, *right* and *bottom row*, *left* and *right*), respectively. The inoculum cell numbers were 420,000 CFU ml^{-1} ($OD_{600} = 0.5$). Disease symptoms were photographed at 3-day (**b**) and 6-day (**c**) post-inoculation

wild-type plants were almost absolutely collapsed, decayed, and eventually dead due to pathogenesis bacteria of soft rot produced large amount of pectic enzymes which degrade plant cell walls. By this time, the percentage of disease susceptible transgenic plants was not increased anymore in both transgenic lines; however, all the susceptible transgenic plants were completely collapsed and decayed as a wild-type (Fig. 4a, b). From this point, the percentage of disease susceptible in both wild-type and transgenic plants were not increased anymore, and the disease-tolerant plants grew well as non-inoculated plants (data not shown). Based on this result, 64.3% of T101, 84.2% of T103 but only 32% of wild-type plants were shown tolerance to soft rot disease; *pin*IISP-*aii* transgenic plants have a significantly enhanced tolerance (2–3-fold) to soft rot disease compared to wild-type plants. Thus, expression of the fusion gene *pin*IISP-*aii* reduces susceptibility to soft rot disease in Chinese cabbage. Transgenic tolerance must be the result of recombinant quorum-quenching enzyme AHL-lactonase activity since this enzyme is encoded by *aii*. The enzyme can effectively quench bacterial quorum-sensing signaling and disintegrate bacterial population density-dependent infections.

A number of quorum-sensing molecules have been identified in both Gram-negative and Gram-positive species. In Gram-negative bacteria, one of the most widespread and best understood families of signal molecules is the AHLs. The AHLs regulate virulence gene expression in a range of plant and animal bacterial pathogens. The AHLs provide global control of exoenzymes production by *Erwinia carotovora* (Fray 2002). *E. carotovora carI* mutants appear to be completely avirulent in a tobacco test system; they can neither macerate plant tissue nor multiply in planta because they lack pectin lyase, pectate lyase, polygalacturonase, cellulose and protease (Jones et al. 1993; Pirhonen et al. 1993). AHL-producing tobacco restored the pathogenicity of an AHL-negative mutant of *E. carotovora* (Fray et al. 1999). The AHLs are highly conserved; they have the same homoserine lactone moiety but differ in the length and structure of the acyl side chain (Dong et al. 2002). But Dong et al. (2000) cloned a novel gene, $aiiA_{240B1}$ coding for an AHL-inactivating enzyme

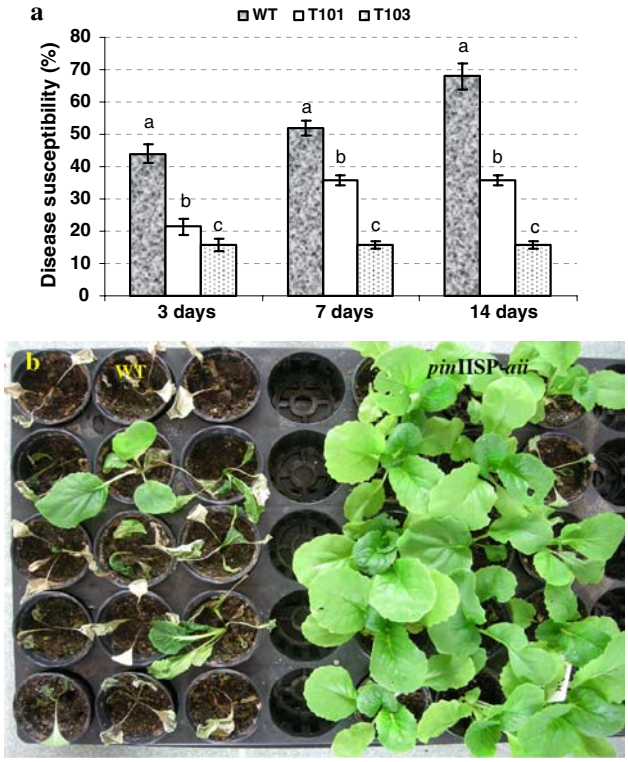

Fig. 4 Evaluation of tolerance against soft rot disease at seedling stage. **a** Disease susceptibility of wild-type (WT) and independent transgenic lines of T101 and T103 at 3-, 7-, and 14-day post-inoculation. *Gray* (WT), *white* (T101), and *dotted* (T103) *columns* indicate the mean of three replicates, and *bars* represent ±SD, respectively. Means indicated with *different letters* (*a*, *b*, and *c* on *top* of the *columns*) were significantly different between each other (ANOVA, Duncan's multiple range test, $P > 0.05$). **b** Wild-type (WT) and *pin*IISP-*aii* transgenic plants are growing after 14-day post-inoculation

($AiiA_{240B1}$), from the Gram-positive bacterium *Bacillus* sp. strain 240B1. The AHL-inactivating enzyme was designed AHL-lactonase, and it is a highly specific enzyme that renders AHL inactive by hydrolyzing its homoserine lactones ring (Dong et al. 2001). Expression of $aiiA_{240B1}$ in transformed *E. carotovora* strain SCG1 significantly reduced release of AHL. Transgenic plants expressing $aiiA_{240B1}$ encoding AHL-lactonase, the gene with high similarity in deduced amino acid sequence to our target gene *aii*, exhibited significantly enhanced resistance to soft rot disease (Dong et al. 2001; Dong and Zhang 2005).

Bacterial soft rot is not only a serious disease for Chinese cabbage, but it often damages all crucifer crops. The pathogen itself is widely distributed in agricultural areas and in uncultivated land (Kikumoto 2000). Pathogen-related loss can be massive, either in the field or during vegetable transport and preservation. Therefore, the control of soft rot disease in the Chinese cabbage industry is of high priority. Here, we could enhance tolerance to soft rot disease in Chinese cabbage inbred line Kenshin by transferring bacterial gene *pin*IISP-*aii* which produced recombinant AHL lactonase enzyme in Chinese cabbage.

So far the transgenic Chinese cabbages have been developed by several groups, but only one of them produced a soft rot disease-resistant transgenic Chinese cabbage (Jung et al. 2008). To the best of our knowledge, the present study is the first to demonstrate the transformation of Chinese cabbage inbred line Kenshin, and the first to describe the effect of the fusion gene *pin*IISP-*aii* on enhancement of soft rot disease tolerance. The approach that we describe has considerable utility for enhancing the breeding of new varieties of Chinese cabbage with anti-soft rot and wide application is greatly recommended.

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