

HETEROLOGOUS EXPRESSION OF ANTIMICROBIAL PEPTIDE LL-37 IN CHINESE CABBAGE WITH ENHANCED RESISTANCE TO PATHOGENS

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ABSTRACT

The human antimicrobial peptide, LL-37 gene was overexpressed in Chinese cabbage 'Osome' (Brassica rapa) by Agrobacterium tumefaciens-mediated transformation. In order to increase the expression of the antimicrobial peptide, we used RolA intron sequence in front of the LL-37 peptide gene. We confirmed the expression of LL-37 in cabbage by RT-PCR and Western Blot analysis. Four transgenic T1 plants were confirmed that LL-37 was expressed. Cabbages expressing the humanLL-37 gene were challenged by various plant pathogen. Transgenic cabbage plants overproducing human LL-37 are expected to possess a durable and wide-spectrum resistance against various pathogens.

KEY WORDS: *Brassica rapa*, Chinese cabbage, *Agrobacterium tumefaciens*, LL-37, resistance, pathogen

INTRODUCTION

Plant pathogens, bacteria and fungi, affect the plant crop products severely. For example, black rot caused by *Xanthomonascampestrispvand* soft rot *Pectobacteriumcarotovorumspcarotovorum* occurs worldwide and causes in severe damage of the plants and reduces yield, especially in Cruciferae. Therefore, the development of Cruciferae resistance to the black and soft rot disease has been a major goal over several decades. In order to decrease the damage caused by bacteria and fungi, lots of antibiotics were applied to agriculture. However, increasing use of the antibiotics caused the occurrence of the resistant gene against the lots of the antibiotics causing the social problem. Recently, recombinant DNA technology,

which introduces disease resistance trait without any alteration of the original genetic background has emerged as an alternative approach to create pathogen-resistant plant. Also, the discovery of the novel antimicrobial activities were recently focused.

In that sense, the antimicrobial peptide can be a good candidate since they have not been reported yet in terms of the occurrence of the resistant gene. Many antimicrobial peptides have been identified to have wide spectrum of antimicrobial activity against fungi or bacteria. Antibacterial peptides are the effector molecules of innate immunity that are composed of 15-45 amino acid residues of the positive net charge. They are found to be about 800 from the animal and plant kingdoms.

Generally, each species has 15-40 peptides made from genes, which code for only one precursor. The action mechanism of the peptides is that they attack membranes to kill bacteria and fungi much faster than the growth rate of the bacteria and fungi (Boman HG, 2003).

In humans, several antimicrobial peptides are found including LL-37. LL-37 is a member of cathelicidin antimicrobial family. LL-37 is released from activated neutrophil granulocytes as a precursor, 18 kDa human cationic antimicrobial protein (hCAP-18). After release, the 37-amino acid α -helical C-terminal end is cleaved off, forming the functional antimicrobial peptide LL-37 (Sorensen et al. 2001). Since LL-37 is the only human antimicrobial peptide in the cathelicidin family, and shows a broad spectrum of antimicrobial

activity at physiologic or elevated salt concentrations, there is a significant interest in developing this peptide for pharmaceutical applications (Reddy et al. 2004; Smet and Contreras 2005; Tjabringa et al. 2003; Travis et al. 2000).

In some plant species, transgenic plants overexpressing foreign antimicrobial protein genes actually acquired resistance to pathogens (Kanzaki et al., 2002). However, antimicrobial peptide-encoding genes have not been explored much yet.

We here report the cloning and expression of LL-37 in Chinese cabbage, *Brassicarapa*. Interestingly, transgenic cabbage plants overexpressing the human LL-37 gene were showed the significant resistance against several fungal pathogens.

MATERIALS AND METHODS

Cloning the LL-37 expression vector

In order to clone 111 bp of LL-37 gene coding for antimicrobial peptide at C-terminal end, we used nested-PCR using following primers with internal EcoRV enzyme sites. To increase the expression of heterologously introduced gene, we used RoIA intron sequence in front of the LL-37 coding region by PCR. The amplified PCR product was subcloned into pGEM-T Easy vector to confirm the nucleotide sequence. In order to express in plant, we

DNA analysis

Total DNA was extracted from various independent transgenic lines as suggested by Manufacturer using a DNeasy Plant Kit (Qiagen, Germantown, MD, U.S.A.).

RNA analysis

Total RNA was isolated from Chinese cabbage using the TRIzol method (Invitrogen, Carlsbad, CA, U.S.A.). In all cases, RNA was treated with RNase-free DNase and the DNase removed according to the manufacturer's instructions (AMBION, Austin, TX, U.S.A.). RNA was quantified in a spectrophotometer at 260 nm. Total RNA (5 μ g) was reverse

Western blot analysis

Total protein was extracted from 0.05 g of leaf tissues of each progeny plant by homogenization in extraction buffer (250mM Tris-HCl, pH7.5, 2.5mM EDTA, 0.1% ascorbic

subclone into plant expression vector, Ti-plasmid VB2 vector. In this vector, LL-37 is under CaMV 35S promoter for constitutive expression. For transformation selection, hygromycin resistance gene was used, and also GFP expression was also used for the transformation. The constructed plasmid was transformed into *Agrobacterium* strain by electroporation, the resulting transformants were stored at -80°C.

Independent LL-37- and HPT- transgenic 'T0' and 'T1' lines were screened by PCR using products generated with the same primers used for LL-37 cloning.

transcribed in 20 μ l reactions using random hexamers (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, U.S.A.), RNaseH-free reverse transcriptase (Fermentas Life Sciences, Burlington, ON, Canada) and GeneAmplifierpAW 109 RNA (2.5 x 10⁵ copies; Applied Biosystems, Foster City, CA, U.S.A.).

acid, 1mM PMSF). The supernatant obtained after centrifugation of the extract at 12,000rpm for 10min was boiled for 10min in sample buffer (extraction buffer containing 0.2% β -

mercaptoethanol, 2% SDS), separated by SDS-PAGE and electro-blotted onto a polyvinylidenedifluoride (PVDF) membrane. Immunodetection was performed essentially according to the method of Matsudaira (1987). Antiserum was purchased from Hbt Ab, clone

Pathogen resistance analysis

Pathogen bioassay was performed on 20 plants from each transgenic plants line and as well as non-transgenic control plants using

number 1-1C12. Detection of the 4-kDa LL-37 protein was carried out with anti-rabbit IgG immunoglobulin (Boehringer, Germany) as the secondary antibody and an ECL developing system (GE Health Care, USA).

Fusariumoxysporum,
Colletotrichumhigginsianum,
Rhizoctoniasolani.

RESULT

Production of transgenic cabbage containing the human LL-37

The 14-Kb plasmid and pl35S-gfp-gus-LL-37 (KJC VB-2) harboring 114 bp human LL-37

cDNA under the control of CMV 35S promoter was used for cabbage transformation (Fig. 1).

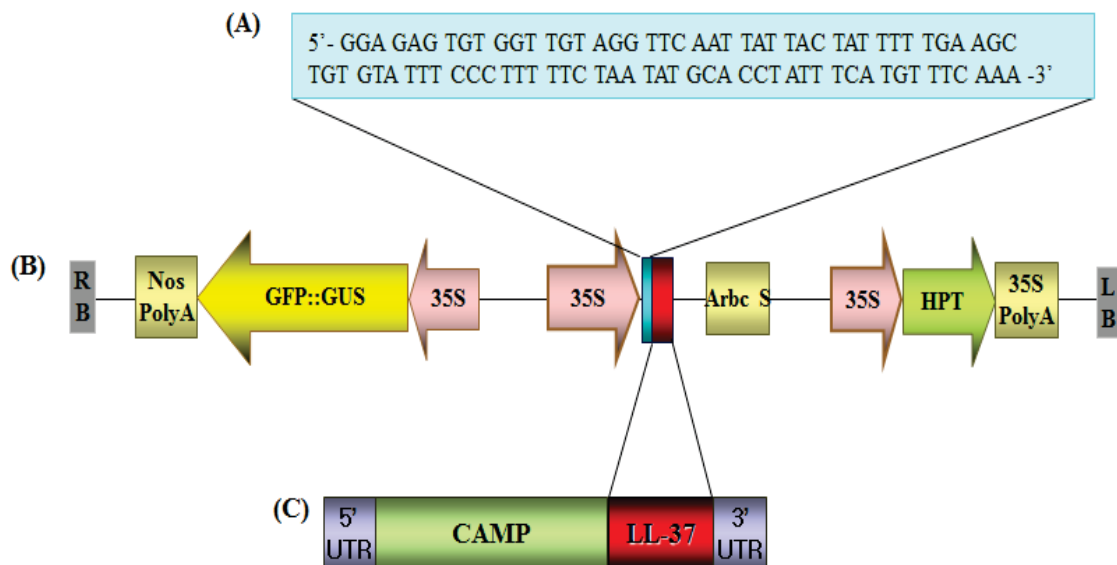


Figure 1. Binary plant expression vector BV2 for the expression of LL-37 gene. (A); transcription enhance regulator region(TERR) obtained from role gene (Gene Bank Accession No. NM-004345).

In addition, in order to increase the expression of the antimicrobial peptide, we used RoLA sequence in front of the LL-37 sequence. After *Agrobacterium tumefaciens* - mediated transformation, transformant cabbages were selected for hygromycin resistance (50mg/L). Transformants started to build up callus after 10 days-culture in the induction medium, an amorphous mass of cells appear from the callus from the late period of the second generation of the culture, and then many multi-shoot appear

from the third generation of the culture (Fig. 2A). After culture in the regeneration medium until the length of the organism reach 7-8 cm, the organisms were induced in order to send out roots (Fig. 2B). The rooted individuals were transferred to the pot to induce the flowering by treating at 8-10°C for 40 days. The flowered individuals were bud pollinated to give offspring T1 (Fig. 2C, D).

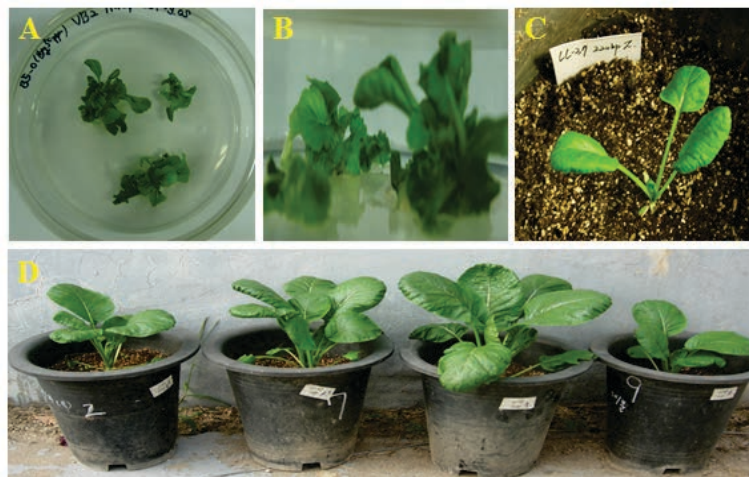


Figure 2. Transgenic plant 'Osome' (*Brassica rapa L.*). The plants transformed with binary vector VB2 including LL-37 gene.

A, Multi-shoot differentiation from Callus formation. B, Regenerated plants in rooting medium. C and D, Acclimation of transgenic plants.

The confirmation of the integration of the LL-37 gene

We confirmed the integration of the LL-37 by PCR using the primers, 35S-specific primer, HPT and LL-37 specific primers using total chromosomal DNA as template. T1 transformants showed PCR product of 300 bp as expected, whereas the control plants did not give the PCR product. In addition, the PCR primers specific for hygromycin resistance

gene also give 713 bp PCR products as expected only in transformants. Also, LL-37 specific primer and RoIA specific primer gave 220 bp PCR products only in transformants (Fig. 3). In conclusion, the transformant cabbages have the integrated LL-37 gene in the chromosome.

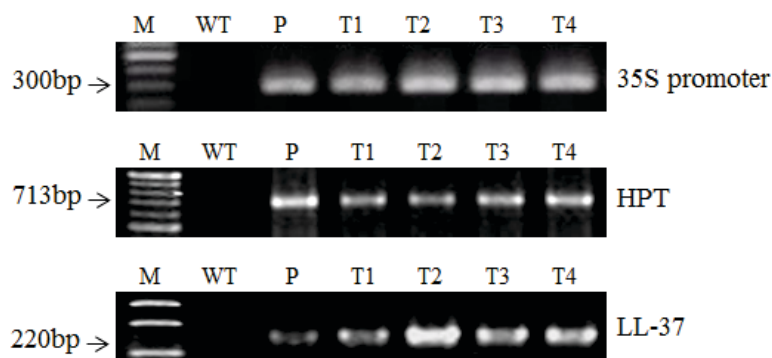


Figure 3. PCR analysis of transgenic 'Osome' (*Brassica rapa L.*) plants using LL-37 gene.

Expression of the LL-37 in the transformants

In order to confirm the expression of LL-37 in the transformants, we performed the quantitative RT-PCR using total RNA from different transformants. We found that transformants expressed LL-37 RNA as expected and the expression levels were similar among the transformants (Fig. 3). Western blot analysis also confirmed that the transformants

expressed the peptides of 4-kD at the various levels specifically detected by the LL-37 antibody, whereas the control plants did not give LL-37 specific band (Fig. 4). Therefore, the heterologous introduced human LL-37 peptide gene was stably integrated in the chromosome to express mRNA to produce LL-37 peptide.

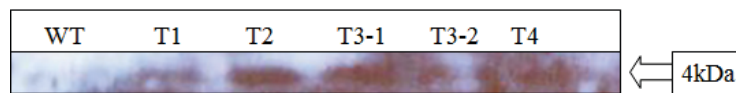


Figure 4. Western blot of transgenic plant Osome (*Brassica rapa*). Plant proteins were transferred to 0.22 micron nitrocellulose membrane, for 2 hours 300mA, membrane was washed by first antibody-human antibody hCAP18/LL-37 (1:10 dilution) and second antibody-ECL rabbit anti-mouse IgG antibody (1:2000 dilution). After reactions, membrane was blotted to X-ray film in dark area. Lane WT: wild type plant, Lane T1 ~ T5: transgenic plants using LL-37 gene.

The increased resistance of the transgenic plants against plant fungi pathogen

The transgenic plant leaves were challenged with several fungal pathogens, *Fusariumoxysporum*, *Colletotrichumhigginsianum*, *Rhizoctoniasolani* to test the resistance of the transgenic plants by wounded and non-wounded fashion. For *F. oxysporum*, the transgenic plant, B21, B22, B23 except B24 showed increased resistance compared to the

control plant. Especially, B21 showed much increased resistance property against *F. oxysporum*. For *C. higginsianum* and *R. solani*. The control plant showed the typical symptom to die 6 days after inoculation, the transgenic plants showed the resistance as shown in Table 1 and Fig. 5. Therefore, we can conclude that the LL-37 peptide expressed in the transgenic plants act as antimicrobial peptide properly.

Table 1

Response of recombinant plant with LL-37 against several fungal pathogens

| Type of plant | Lesion size (cm) when inoculated with | | |
|---------------|---------------------------------------|------------------------------------|---------------------------|
| | <i>Fusarium oxysporum</i> f. sp. | <i>Colletotrichum higginsianum</i> | <i>Rhizoctonia solani</i> |
| WT | 1.8 | 1.2 | 12.0 |
| T1 | 0.2 | 0.3 | 0.2 |
| T2 | 0.9 | 0.6 | 3.0 |
| T3 | 0.7 | 0.1 | 6.0 |
| T4 | 1.4 | 0.2 | 0.5 |

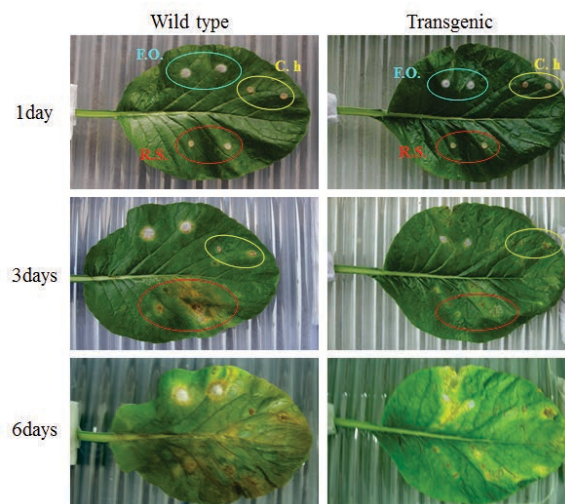


Figure 5. Disease test of several fungal pathogens in transgenic *Brassica rapa* ('Osome') plants. Inoculation fungal upon leaves of transgenic plants using LL-37 gene.

F.O: *Fusariumoxysporum*f. sp.
 C.h: *Colletotrichumhigginsianum*
 R.S: *Rhizoctoniasolani*

DISCUSSION

Living individuals have developed various defense mechanisms against foreign pathogens as well as environmental stresses. Upon the attack of pathogens, a signal is generated to transmit downstream and to turn on a number of defense systems including antimicrobial proteins expression to fight off pathogens. LL-37, a protein belonging to the cathelithidin family, is one of such antimicrobial proteins expressed in human as an innate immunity system. Here, we expressed LL-37 in Chinese cabbage transgenic plants inhibited the growth of several fungi. LL-37 seems to increase the permeability of bacterial and fungal membranes (Thevissen et al. 1999).

We used the CMV 35S promoter to drive the expression of the LL-37 gene. This promoter is extremely strong in monocots (Christensen et al. 1992) and is expected to constitutively produce human LL-37 in transgenic Chinese cabbage plants. These transgenic plants were overproducing human LL-37 proteins (Fig. 5). It is obvious that the overproduction of human LL-37 was the casual factor for the enhancement of disease resistance. In these plants, human LL-37 should also inhibit the growth of fungal hyphae after their invasion of the epidermis cells.

Since pathogens can overcome true resistance in the field by mutations in the cognate avirulence genes, any transgenic resistance

based on true resistance genes may easily break down. On the other hand, this problem probably does not occur in transgenic plants overexpressing antifungal genes. Therefore, the overproduction of the human LL-37 peptide with antifungal activity is expected to confer durable resistance against a wide variety of Chinese cabbage. In addition, transgenic Chinese cabbage harboring the human LL-37 gene may additionally have enhanced resistance against diseases caused by other microbes. To obtain a high level of resistance as observed in the R-gene mediated resistance, overexpression of multiple antifungal proteins with different functions may be necessary. The performance of tobacco plants co-expressing the barley transgenes, a class-II chitinase, a class-II β -1,3-glucanase and a type-I ribosome-inactivating protein, in a *Rhizoctonia solani* infection assay was reported to reveal significantly enhanced protection against fungal attack when compared with the protection levels obtained with corresponding isogenic lines expressing a single barley transgenes at a similar level (Jach et al. 1995). Transgenic plants harboring either other antimicrobial gene or multiple ones as well as the human LL-37 gene, recombined by crossing of different transgenic plants, will inhibit the growth of pathogen at an even higher level and with increased durability.

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