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Transfer and expression of the rabbit defensin *NP-1* gene in lettuce (*Lactuca sativa*)

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ABSTRACT. Lettuce (*Lactuca sativa* L.) is an annual plant of the daisy family, Asteraceae, with high food and medicinal value. However, the crop is susceptible to several viruses that are transmitted by aphids and is highly vulnerable to post-harvest diseases, as well as insect and mammal pests and fungal and bacterial diseases. Here, the rabbit defensin gene *NP-1* was transferred into lettuce by *Agrobacterium*-mediated transformation to obtain a broad-spectrum disease-resistant lettuce. Transgenic lettuce plants were selected and regenerated on selective media. The presence of the *NP-1* gene in these plants was confirmed by western blot analyses. Resistance tests revealed native defensin *NP-1* expression conferred partial resistance to *Bacillus subtilis* and *Pseudomonas aeruginosa*, which suggests new possibilities for lettuce disease resistance.

Key words: Lettuce; Transgenic plants; Rabbit defensin gene *NP-1*

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a globally important leafy vegetable that is widely grown and easily cultivated, although it requires relatively low temperatures. Lettuce is low in calories and rich in lactucin and mannitol (Song et al., 2014). Lactucin is an ingredient of lactucarium that is found in some varieties of lettuce, and has been shown to have analgesic and sedative properties (Wesołowska et al., 2006). Mannitol is used in osmotherapy to reduce acutely raised intracranial and intraocular pressure, and also plays a key role in the treatment of brain edema and glaucoma (Rabinstein, 2006). Currently, the quality and yield of lettuce is severely constrained by plant diseases such as *Lactuca sativa* Sclerotinia and *Pseudoperonospora cubensis*. *Lactuca sativa* Sclerotinia is widespread in lettuce plantations globally, and causes considerable damage to the basal part of stems and leaves (Waipara, 2006; Chitrampalam et al., 2010).

There has been considerable research, locally and globally, to improve the disease resistance of lettuce. In addition to traditional breeding methods, such as disease resistant plant seed selection, research on cultivating resistant transgenic plants using molecular methods has recently increased. Zhang et al. (2010) transferred the *MI-1* gene into lettuce, and the T1 generation transgenic plants showed resistance to root-knot nematodes. Dias et al. (2006) transferred the decarboxylase gene (*oxdc*) into lettuce, and RT-PCR analysis carried out with *Sclerotinia*-resistant lines indicated the expression of *oxdc* gene transcripts. Yoichi et al. (2006) transferred the *LBVaVCP* gene into lettuce and found that this line had resistance to both the big-vein associated virus and Mirafiori lettuce virus. Subsequently, the *MiLVCP* gene transferred into lettuce with resistance to big-vein disease was also obtained, and this was the first report of an inserted nucleotide sequence derived from the genus *Ophiovirus* to create virus-resistant plants (Yoichi et al., 2009). Therefore, this research indicated that it was possible to use transgenic lettuce as a resistant cultivar or breeding source.

Rabbit defensin (NP-1) has significant inhibiting or killing effects on pathogenic microorganisms such as Gram-positive and -negative bacteria, fungi, viruses, and even some malignant cells (such as tumor cells and HIV), which was determined by antibacterial tests *in vitro* (Zhou et al., 2011). As a new broad antibacterial spectrum defensin, it possesses far-reaching implications in plant transformation. The mechanism of rabbit defensin (NP-1) determined that it was difficult to target microorganisms to yield resistant mutations. Recently, rabbit defensin (NP-1) has been transformed into some plants and expressed via genetic engineering (such as the pollen tube pathway and *Agrobacterium* mediation), which plays an important role in the genetic engineering of anti-disease plants and plant species improvements (Zhou et al., 2011).

The aim of the present study was to successfully apply an *Agrobacterium*-mediated transformation strategy to obtain transgenic lettuce that could stably express *NP-1*. Simultaneously, we established effective test methods, which accurately and stably determined the biological activity of the transformed plants, and laid the foundation for cultivating transgenic lettuce with broad-spectrum disease resistance. The *NP-1* gene was applied in two sequential transformation steps to develop transgenic lettuce.

MATERIAL AND METHODS

Bacterial strains and plasmids

The *A. tumefaciens* bacterial strain harboring the plasmid pBinU-Omega-HisNP1

(Figure 1) was used for transformation of lettuce. The binary plasmid, pBinU-Omega-HisNP1, gifted by the Chinese Academy of Science Institute of Genetics and Developmental Biology, was constructed by inserting a 130 bp fragment harboring the *NP-1* gene under the Ubiquitin promoter and NOS terminator. The *NP-1* gene upstream of the His-NP1 sequence contained six His-tags, and a small 2.3-kb fragment that could be recovered by *Hin*dIII and *Sac*I double digestion from the plasmid. The transconjugants were selected on LB medium with 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin, and the presence of plasmids was confirmed by PCR analysis.

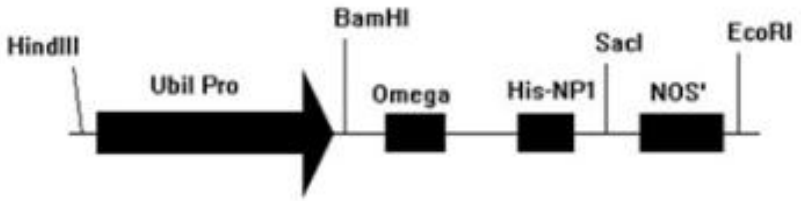

Figure 1. The T-DNA region of the plasmid, pBinU-Omega-HisNP1, used for lettuce transformation. Nos, polyadenylation and termination signals; His-NP1, rabbit defensin NP-1 coding sequence; Ubi Pro, Ubiquitin promoter.

Plant material and transformation

Mature seeds of the Zhengyuan Italy lettuce, purchased from the Vegetable and Seed Center of Yichang were used to produce cotyledons, which were used for shoot regeneration on the shoot induction medium [MS salts (Murashige and Skoog, 1962), 0.5 mg/L 6-Benzylamino Adenine (6-BA), 0.3 mg/L Naphthylacetic Acid (NAA), 30 g/L sucrose, and 8 g/L agar, pH 5.8]. *Agrobacterium* was grown on LB medium at 28°C in a shaker (180 rpm) until an optical density of 0.4 (OD_{600}) was reached. Bacterial cells were pelleted by centrifugation at 10,000 *g* for 10 min at 28°C and re-suspended in an equal volume of MS medium supplemented with 100 μM acetosyringone (AS). The cotyledons were excised and pre-incubated on the medium for 2 days and then infected by swirling in the *Agrobacterium* culture for 10 min. The infected cotyledons were co-cultivated on the co-cultivation medium (MS salts, 0.5 mg/L 6-BA, 0.3 mg/L NAA, 30 g/L sucrose and 8 g/L agar, 100 μM AS, pH 5.6). After 2-3 days, the cotyledons were placed on the selection medium supplemented with 75 mg/L kanamycin (Kan) and 500 mg/L carbenicillin (Carb) to prevent *Agrobacterium* growing excessively. When adventitious buds grew to 1-2 cm, they were transplanted to the 1/2 MS medium with 0.1 mg/L Indoleacetic Acid (IAA). Finally, the transgenic plants were acclimatized and grown in a greenhouse.

PCR analysis of the transgenic lettuce plants

The genomic DNA of transgenic and wild-type lettuce were taken from leaves and extracted using the CTAB method. PCR was performed using primer pairs specific for the coding sequence of NP-1 (Forward: 5'-GCCATCATCATATGGTTGTTTGTGCA-3', Reverse: 5'-GCGAGCTCTTAACGACGACAACAAAGTGG-3'). The PCR cycle conditions were: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 35 cycles followed by an initial denaturation at 94°C for 3 min, and a final extension cycle at 72°C for 10 min. The PCR products were separated on 2% (w/v) agarose gel to show whether the 130 bp specific bands existed.

Southern blot analysis

Plant DNA from control and transgenic lettuce plants were digested with restriction enzymes (*Hin*dIII and *Sac*I, the resulting fragments were electrophoresed in 0.8% agarose gel (Agarose II, Sigma, USA) and detected by ethidium bromide staining. After depurination and denaturation, DNA was transferred to a nylon membrane (Boehringer Mannheim). The DNA probe was labeled with Digoxigenin (DIG) (Boehringer Mannheim, USA). Hybridization was performed at 42°C overnight. The Hybond-N+ membrane was washed using 2X SSC buffer containing 0.1% SDS for 5 min (twice) at 25°C, then washed using 0.5X SSC buffer containing 0.1% SDS for 15 min (twice) at 68°C. The immunological detection of the DIG-labeled probe was performed using a DIG Wash, Anti digoxigenin-AP conjugate (Roche), and Block Buffer Set (Roche).

Western blot analysis

The expression of the target gene was detected by western blotting. The total protein from control and transgenic lettuce plants that showed a hybridization signal in the Southern blot was extracted using a Plant Protein Extraction Kit (ComWin, China). The protein samples were separated on a 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel and electro-transferred onto a Polyvinylidene Fluoride (PVDF) membrane in an electro-blotting transfer buffer. The membranes were blocked with 5% non-fat milk powder in Tris Buffered Saline Tween (TBST) with gentle agitation overnight to prevent non-specific antibody reaction. The membrane was incubated at 25°C for 2 h in a 1:800 dilution of Anti-His Tag Monoclonal Antibody (ComWin, China) in a TBST antibody dilution buffer and washed five to six times with TBST buffer. Then, the membrane was incubated for 2 h in a 1:50,000 dilution of goat anti-mouse antibody labeled with horseradish peroxidase (Boster, China) in TBST buffer and washed five to six times using TBST buffer. Antibody binding was detected by incubation with an ECL substrate solution, following the manufacturer's instructions. An image analysis of the western blot was conducted using a Kodak Imaging Station (Kodak, USA).

In vitro antimicrobial assay

The anti-microbial activity of protein from transgenic lettuce extracts was tested using agar plates. Each plate contained four wells; three wells were filled with equal volumes of protein from the transgenic lettuce, and the other one well was filled with the same volume of protein from the wild-type lettuce. Liquid cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa* were spread on the agar plates and grown under appropriate conditions. After a period of microbial proliferation, growth inhibition was observed and photographed as the appearance of clear zones.

RESULTS

Agrobacterium-mediated lettuce transformation

The seedlings were cut at five days (Figure 2A). After *Agrobacterium*-mediated transformation, kanamycin-resistant adventitious buds were induced into a clump shoot on the

selection medium after 3-4 weeks of culture (Figure 2B). The transgenic shoots regenerated, whereas those of the control group was induced into a bud, gradually became brown, and died. After these 1-2 cm long transformed shoots were transferred to 1/2 MS with 0.1 mg/L IAA, complete plantlets were developed (Figure 2C) and rooted (Figure 2D). The transgenic lettuce was transplanted to a greenhouse for acclimation (Figure 2E); however, some abnormal plantlets died during acclimation in the greenhouse.

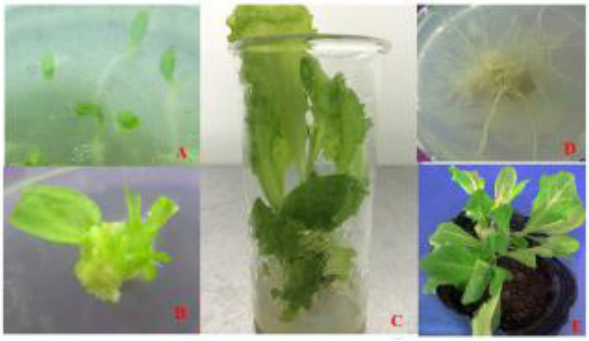

Figure 2. Acquisition of transgenic lettuce plants: **A.** 5-day-old seedlings; **B.** adventitious buds; **C.** complete plantlets; **D.** rooting of resistant buds; **E.** transplanted resistant plants.

PCR analyses of regenerated lettuce plants

Total DNA samples were extracted from the leaves of regenerated lettuce plantlets with kanamycin resistance. The results of the PCR analysis of the transgenic and non-transgenic lettuce DNA samples are shown in Figure 3. DNA samples from the regenerated plants and positive control plasmids showed amplification products with an expected size (130 bp), whereas there was no signal in non-transgenic plant DNA. The PCR experiments initially suggested that the *NP-1* sequences were presented in the genome of most of the regenerated lettuce plants.

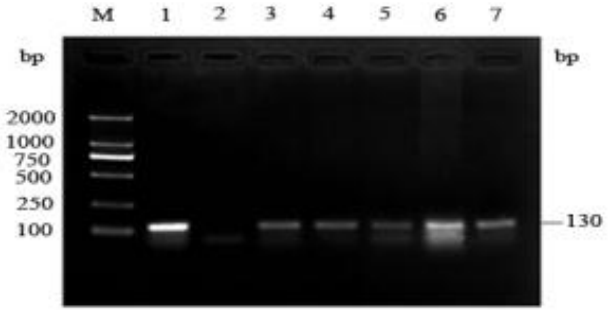

Figure 3. PCR analysis of regenerated lettuce plants: DNA marker (*lane M*), positive control plasmid pBinU-Ome-HisNP1 (*lane 1*), and an untransformed plant (*lane 2*). A 130-bp PCR fragment was found both in the genomic DNA (*lanes 3-7*) and positive control plasmid.

Southern blot analysis

The integration of the *NP-1* gene into lettuce with amplified PCR target fragments was

confirmed by Southern blotting. In Southern blot hybridization experiments, DNA samples from the regenerated lettuces hybridized with the probe derived from the *NP-1* gene (Figure 4A), and the intensities of the hybridization signals differed among the various DNA samples. Therefore, the DNA samples with strong signals were analyzed further. Hybridization signals were observed in the transgenic lettuce lines; one insertion site of the *NP-1* gene was detected in these lines, which confirmed that the *NP-1* gene was transmitted stably to transgenic lettuce (Figure 4B). From the results, it can be seen that different hybridization signals were obtained, even in the same DNA samples.

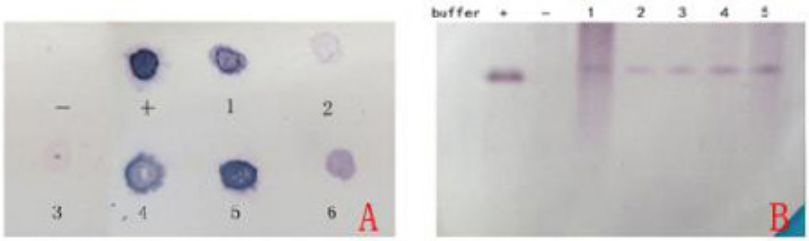

Figure 4. A. Southern blot analysis of genomic DNA from transformed lettuce plants. **A.** *Lane* - = negative control, *lane* + = positive control, *lanes 1-6* = transgenic plants; **B.** *lane* + = positive control, *lane* - = negative control, *lanes 1-5* = transgenic plants. Southern blot analysis (**B**) of plant genomic DNA products from control and transgenic lettuce plants were digested with restriction enzymes (*Hin*dIII and *Sac*I).

Western blot analysis

In the present study, monoclonal antibodies labeled with anti-His were selected as the primary antibodies and goat anti-mouse antibodies labeled with horseradish peroxidase as the second antibodies. The presence of protein crude extracts of transgenic lettuce with Southern blot hybridization signals were detected by western blotting, which showed hybridization signals in the Southern blot. There was no protein signal in wild-type lettuce plants. The size of the target protein monomer was about 3.9 kDa. The positive bands appeared in the position of about 40 kDa, and lanes 1-6 were also located in the position of about 40 kDa (Figure 5), which demonstrated that the target protein existed in the form of a polymer in a water solution and the target gene was expressed preliminarily in plants.

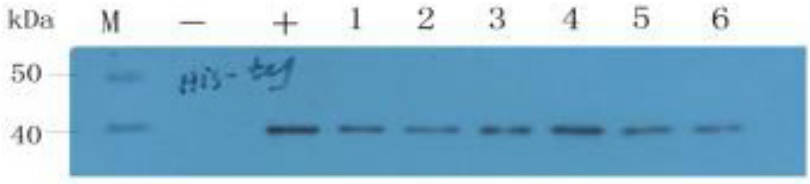

Figure 5. Western blot analysis of transgenic lettuce plants. *Lane M* = marker, *lane* - = negative control, *lane* + = positive control, *lanes 1-6* = transformed lettuce.

In vitro biological activity assays

Crude protein extracts from transgenic and non-transgenic lettuce were used for anti-microbial assays with *B. subtilis* and *P. aeruginosa* bacteria (Figure 6), Figures 6A and 6B

represent the transformed sample and negative control; the inhibition zones of samples 1-3 in *B. subtilis* were smaller than those in *P. aeruginosa*, whereas the non-transgenic plants did not form an inhibition zone, which initially confirmed that the *NP-1* gene was an expressed protein in transgenic lettuces.

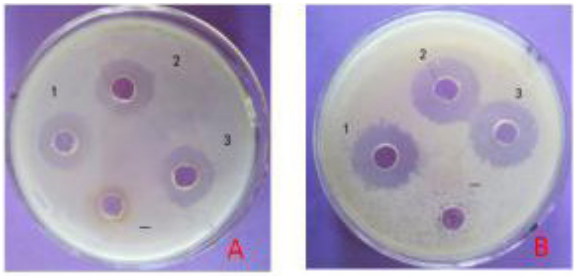

Figure 6. *In vitro* microbial activity against *Bacillus subtilis* (**A**) and *Pseudomonas aeruginosa* (**B**). The protein of transgenic lettuces (1, 2, 3) showed antibacterial activity, whereas the control (-) had no antibacterial activity.

DISCUSSION

Since the first transgenic tobacco plant was developed in 1983, plant genetic engineering has made remarkable achievements. Lettuce has emerged as a valuable organism for transgenic plant research, and exogenous gene expression is stable and highly inherited in lettuce progenies (Song et al., 2014). However, the transfer of the rabbit defensin *NP-1* gene into lettuce has not yet been reported to our knowledge. In this study, the universal method of *Agrobacterium*-mediated transformation was modified and used to express the rabbit defensin *NP-1* gene in lettuce. Successful transgenesis could be easily influenced by *in vitro* plant regeneration systems (Thirukkumaran et al., 2009; Zhao et al., 2011). Therefore, the lettuce regeneration system was optimized with an approximately 70% regeneration rate of cotyledon explants. In addition, phenolic compounds are helpful to improve the conversion efficiency. Xu et al. (1988) first demonstrated that the excitation and efficient expression of the virulence (*Vir*) gene, which is necessary for the Ti plasmid of the *Agrobacterium* transfer into the host cell, was activated by AS trauma-induced molecules. Lai et al. (2006) identified 11 AS-induced proteins, which were implicated in *Agrobacterium* virulence. Therefore, 100 μM AS was added in this experiment.

Appropriate antibiotics ensured high efficiencies of target transgenic plant screening (Wang et al., 2006). In the present experiment, the neomycin phosphotransferase (*NPTII*) gene, which can phosphorylate kanamycin hydroxyls, was used as a selective marker. An antibiotic sensitivity test showed that the optimal concentration for kanamycin and the bacteriostatic agent, carbenicillin, were 75 mg L^{-1} and 500 mg L^{-1}, respectively. However, many kanamycin-resistant plants failed to amplify target gene fragments in the PCR analysis, which suggested there were some false transformants.

The maize Ubi1 promoter is more effective than the CaMv35S promoter and has a low copy number of genes foreign to plants (Chen et al., 2001a; Xu et al., 2004). In this report, the rabbit defensin (*NP-1*) gene was driven by the Ubi1 promoter in lettuce. As expected, PCR showed that a unique band of 130 bp in length was amplified in transgenic plant genomes; and the Southern blot analysis showed a 2.3 kb *NP-1* gene fragment from regenerated plants after

hybridization, which is consistent with previous research (Li et al., 2004), further evidence that the *NP-1* gene was transferred into the lettuce. Western blot analysis detected the presence of the NP-1 fusion protein in lettuce. The size of the target protein monomer was about 3.9 kDa, but the target protein of six samples detected in this experiment was about 40 kDa, which demonstrated that the target protein existed in the form of a polymer in a water solution. The six histidine (His) residues added upstream in the *NP-1* gene combined with the target protein to form a dimer or polymer (Li et al., 2004). This indicated the target gene was expressed in plants.

The antibacterial activity *in vitro* would be helpful in confirming the expression of foreign genes in lettuce. Results showed that the transformed plants inhibited *B. subtilis* and *P. aeruginosa* growth, which was consistent with the results of *NP-1* genes introduced into poplar and *Chlorella* (Zhao et al, 1999; Wang et al., 2001). The *NP-1* disease-resistant mechanism was combined with a virus coat protein to lose their biological activity (Chen et al., 2001b). However, only three strains showed better inhibitory effects in six positive transgenic plants, which may be owing to the following: first, the low expression of the *NP-1* gene; there is a threshold level of transgene-derived transcripts, and disease-resistant mechanisms cannot be triggered until the threshold level in transgenic plants is reached (Fu et al., 1998); second, the genetic variation of other disease-resistant related genes during the process of transformation; and third, false positives.

In conclusion, we developed lettuce plants transformed with the *NP-1* gene, and confirmed that the exogenous gene was quantitatively expressed in plants and showed positive bacterial inhibition. Presently, research on *NP-1* is focused on its disease resistance in transgenic plants, and further research work should be performed on its medical applications.

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