Integrating the hrap Gene from Sweet Pepper into Potato Enhances Resistance to Phytophthora infestans

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Abstract
Phytophthora infestans, also known as potato late blight, is a serious and widespread disease of potato that causes significant yield losses in potato production every year. Controlling this destructive disease has become a great challenge for potato farmers. In order to minimize commercial losses and improve tuber quality, it is a smart way to build potato disease resistant capacity by the path of transgenic genetic improvement. In this present study, a gene encoding the hypersensitive response-assisting protein (hrap) originally isolated from sweet pepper induced with the harpin Pss-mediated hypersensitive response (HR) was introduced to the potato variety Burbank mediated by Agrobacterium tumefaciens. The rate of transgenic acquisitions was 61.15% positive detected by PCR, whereas 53.85% of the PCR positive plants were validated by southern blot assay. RT-PCR analysis showed that the hrap gene was integrated into the potato genome and expressed in the transgenic potatoes. The sequence of the hrap gene isolated from transgenic potato was 99.3% similarity identified with that of the hrap genes deposited in GenBan (GenBank: AF168415). The resistant assay was performed by artificially incubating the pathogens of P. infestans. The results showed that the transgenic potatoes exhibit the resistant enhancement.

Keywords
Potato; Hrap gene; Phytophthora infestans Agrobacterium; mediated transformation

Background
China is recognized as the world’s largest producer of potatoes (Solanum tuberosum L.) with over 490 million hm² in cultivation based on calculation of perennial cultivating basis. Guizhou, a province located in Southwest China, is deemed to be the largest producer of potatoes in this country in 2006, with a planting area of 590,000 hm² (Source from: China Agricultural Yearbook in 2007). Potatoes are subject to a variety of potential diseases, such as Phytophthora infestans also called as late blight, which is responsible for significant historical crop losses, the most notorious of which was the Irish potato famine in the 19th century. If this crop is infected with late blight, the yield of potato will lose with poor quality, irregular shape and appearance. It was reported that economic losses related to infection of P. infestans totally are more than 1 billion U.S. dollars in China every year. Definitely, P. infestans is also a particular problem in the subtropical, high-humidity areas of Guizhou as well as the neighbor, Yunnan provinces (Song et al, 1996), where temperatures are mild and rainfall are high. Based on the basic geographic and climate data, the International Potato Center (CIP, Lima, Peru) completed computer simulations of China's potato production, incorporating the characteristics of P. infestans infection, and concluded that the fungicide spray times need at least nine times in Southwest China higher than that in other potato-growing regions of the country, the actual fungicide spray times of the latter were only five times for P. infestans control (He et al, 1999). Unfortunately, with the increasement of the chemical application, the pathogen had acquired the pesticide resistances and there is a serious public concern arisen due to the direct
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and indirect pollution effects of pesticides on non-target insects and animals, including humans. Regarding these public and environmental concerns, the identification, development and utilization of P. infestans-resistance genes are important priorities that might greatly benefit the potato breeding and production.

Recent studies of the mechanisms in the fields of plant disease resistance and pathogenicity had identified some protein factors related to plant defense responses. The resistant ability to P. infestans can be improved by transforming the pathogenesis-related (PR) gene in potatoes. It is reported that transgenic potato plants carrying the tobacco osmotin protein were able to delay the appearance of P. infestans spots and reduce the frequency of P. infestans infection, which indicated an improved capacity in late blight resistance in the plant leaves (Liu et al (1994), Zhu et al (1996) and Li et al (1999a)). Jin et al (2001) introduced the thaumatin protein gene (tlp) with the selective marker of the herbicide-resistant bar gene into a potato plant mediated by Agrobacterium tumefaciens to delay onset of late blight symptoms following inoculation with late blight spores. Yang et al (2001) introduced the gene, avrD, to potato plants and confirmed that disease resistance was enhanced while infected with the Phytophthora protein, elicitin. Transgenic potato plants haeboring the glucose oxidase gene (go) can accumulate H2O2 inside the plant and acquire distinct resistance to P. infestans (Zhang et al., 2001). Nan et al (2006) also reported that the transgenic potato plants with the bean chitinase gene improved the anti-pathogen characteristics more than 30% over the control group without the chitinase gene.

The hypersensitive-response assisting protein (HRAP), identified in sweet peppers (Capsicum annuum cv. ECW), might intensify the harpinPss-mediated hypersensitive response (HR), which a defense mechanism often found in plants exhibiting disease resistance. The hrap gene encoding the HRAP can split the harpinPss polymer into monomer and dimmer components, the later will cause much more elevated allergic necrosis and retarding the propagation of bacterial spot virus and the bacterial pathogen, Xanthomonas, in sweet pepper, resulting in alleviating symptoms (Chen et al, 1998 and 2000; Ger et al, 2002). For example, the hrap gene was introduced into tobacco, the resulting transgenic plants were acquired resistant to tobacco wildfire and soft-rotting bacteria (Ger et al, 2002). Similarly, Pandey et al (2005) introduced hrap into Arabidopsis thaliana mediated by Agrobacterium to generate transgenic plants with resistant to soft-rotting bacteria. In present study, the hrap gene was introduced into potato cultivar by Agrobacterium mediation and the resistant phenotypes of the transgenic plant were evaluated.

1 Results

1.1 The hrap gene introduced into potato mediated by Agrobacterium

The transformation was performed using an Agrobacterium tumefaciens standard binary (T-DNA and vir regions) vector system, tissue cultured potato leaflets as explants. Callus was observed two weeks after the leaflets was cultured in the callus induction medium. The callus induction rate was over 80%. The adventitious buds were observed after the calli were transferred into SIM for 3~4 weeks. The bud induction rate was 50%. About ten days after the adventitious buds were transferred into rooting medium, regenerated plantlets were obtained. Finally, we had obtained over 20 transgenic lines, more than 400 transgenic plants, and over 100 transgenic potatoes.

1.2 Anti-Kan rooting-selection

The non-transformed adventitious buds (control) grew roots only in the medium with Kan free, they exhibited only a small number of aerial roots in the MS-Kan medium (Table 1). However, the transformed adventitious buds readily grew roots in the MS-Kan media. These data showed that the MS medium containing 75 mg/L Kan was suitable for the rooting-selection of transgenic plants (Figure 1). The rooting-rate of the transformed adventitious buds was conducted twice. The rooting rate of the transformed adventitious buds was calculated twice. The rooting rate of the transformed adventitious buds in the Kan medium was 76.32% and 86.21%, respectively (Table 2). The second rooting rate was about 10% higher than the first, which indicates that as for Burbank, twice rooting-selection is efficient and imperative.
Table 1 Effect of Kan on the root growth of adventitious buds

| Kanamycin dose (mg/L) | Non-transformed adventitious buds | Transgenic adventitious buds |
|-----------------------|----------------------------------|-----------------------------|
| 0                     | Normal rooting and robust growth  | All were normal in rooting, grew robustly, roots were long and more |
| 50                    | No roots, normal color in branches and leaves | Grew roots partially, with 5–8 roots |
| 75                    | No roots, stunted plants          | Grew roots partially, with 3–4 roots |

Table 2 Rooting rate of transformed adventitious buds

| Selection          | No. of adventitious buds inoculated | No. of adventitious buds rooted | Rooting rate  |
|--------------------|------------------------------------|---------------------------------|---------------|
| First selection    | 38                                 | 29                              | 76.32%        |
| Second selection   | 29                                 | 25                              | 86.21%        |

Figure 1 Effect of different Kan concentrations on the root growth of adventitious buds

1.3 PCR detection

The PCR positive rates of the transformant lines and plants were 93.75% and 61.15% respectively (Table 3). Results of PCR electrophoresis of partial transformant plants are shown in Figure 4. Both positive plants and positive Agrobacterium could be amplified with a band of approximately 720 bp, while the non-transformed plants had no target band (Figure 2).

1.4 Southern Blot analysis

The lines and plants detected positive by PCR assay were selected for Southern Blot assay. Of the 13 plants detected, seven showed positive response, yielding a rate of 53.85% (Figure 3), which indicates that the target gene had been successfully transformed into the potato genome.

Table 3 PCR analysis of transgenic lines and plants

| No. of lines tested | No. of positive lines | Rate of positive lines | No. of plants tested | No. of positive plants | Rate of positive plants |
|---------------------|-----------------------|------------------------|----------------------|------------------------|-------------------------|
| 16                  | 15                    | 93.75%                 | 157                  | 96                     | 61.15%                  |
Figure 3 Southern Blot analysis of some transgenic plants
Note: 1: Positive control, plasmid Pbhrap, the relatively dark color was a result of poor banding; 15: Negative control, an un-transformed plant; 2~14: Transgenic plants; Bands of 2, 3, 4, 6, 9 and 10 are weak, meaning the hrap gene was not for sure transferred into plants; Lines 5, 7, 8, 11, 12, 13 and 14 were considered to be positive transgenic plants

1.5 RT-PCR analysis
RT-PCR analysis was performed on 10 transgenic generation plants randomly selected from different lines in order to verify the expression of transgenic plants. No PCR product was detected in the negative control (non-transformed) plant (Figure 4, lane 11). All the 10 tested plants were confirmed positive by RT-PCR using nested gene-specific primers with an expected 720 bp fragment detected (Figure 4, lanes 1~10), confirming that the hrap gene was stably integrated into the genome and expressed in these plants.

1.6 Sequencing validation
DNA sequencing analysis was performed by comparing the hrap genes isolated from transformation plants in this experiment with that registered in the Genebank (GenBank: AF168415). Results demonstrated that there of the ten transgenic plants were five bases different from AF168415, and a similarity of 99.3%. (Nos of 226, 296, 438, 534 and 583) was detected.

1.7 Assay of disease resistance
According to Center International Potato (CIP) late blight 9-score standards, the disease resistance to P. infestans detection in transgenic lines indicated a resistant line rate of 50%. Five plants (1-2-23, 1-2-32, 1-4-8, 1-10-9, 1-14-26) among the 42 tested plants showed resistance to P. infestans, and the rate of resistant plants was calculated to be 11.90% (Table 4).

According to the earliest wilting leaves time and whether the entire plant wilted as disease resistance and sensitivity standards, both the rates of resistant lines and resistant plants to R. solacearum were 50%. Two transgenic plants (1-4-5, 1-10-7) were highly-resistant to R. solacearum, and 1-1-27 was moderately resistant to the disease, while the control was susceptible (Table 4).

Table 4 Detection of disease (P. infestans and R. solacearum) resistance in transgenic potatoes

|                | No. of detections | No. of plants (lines) of resistance | Rate of resistance |
|----------------|-------------------|------------------------------------|-------------------|
| P. infestans   | Lines 8           | 4                                  | 50.00%            |
|                | Plants 42         | 5                                  | 11.90%            |
| R. solacearum  | Lines 6           | 3                                  | 50.00%            |
|                | Plants 6          | 3                                  | 50.00%            |

2 Discussion
In the analysis of the rooting ability of transformed adventitious buds on MS medium containing 75 mg/L Kan, about 24% of the buds could not grow root after the first rooting-selection, and 14% after the second rooting-selection. These results indicated that the twice rooting-selection of the transformed plants was effective. The results are consistent with the data reported by Zhang et al (2004).

In this study, PCR positive rate of transgenic plants obtained was 61.15%, which was similar to the rate obtained by transforming HarpinEa gene into Atlantic (55.14%) reported by Li et al (2002). However, Su et
al (2004) transferred Bt gene into Atlantic, only with a positive rate of only 2.96%. In this experiment, we observed both positive and negative PCR results from different plants propagated by one line. It is possible that the inserted exogenous gene was lost over the course of multiple asexual propagations, or the exogenous gene expression was blocked in the transformant, or the PCR amplification stripe was simply too weak to be discerned using the methods employed in this study. Regenerative plants may differentiate from a number of cells, some of which may be non-transformed, thus passing those characteristics on to the regenerated plants.

Multiple analyses employed in the current investigation, i.e., PCR, Southern Blot, RT-PCR and sequencing, illustrate the successful integration and expression of the target gene in transformed plants.

Li and Fan (1999b) identified P. infestans resistance in 55 transformed plants with Harpin protein, and found that two were highly resistant. Zhang et al (2001) reported that transformed go gene regenerative plants exhibited a delayed occurrence of late blight symptoms. Li et al (2002) screened seven lines (out of 24 positive PCR lines) that demonstrated a significant difference in disease resistance compared to the control. Wang et al (2007) reported that hrap gene, integrated into a tomato plant, improved the plant’s resistance to R. solacearum. In this study, the hypersensitive-response promotion protein gene hrap was introduced into potato, resulting in transgenic potato plants that were resistant to P. infestans and R. solacearum. The experimental results indicate that transformation of hrap gene into potato to improve the ability of disease resistance is feasible.

The integration of the pathogenesis-related (PR) protein gene is considered to be significant in producing disease resistant plants and in improving the quality and yield of potato.

3 Materials and Methods

3.1 Plant materials

The tested potato cultivar called Burbank, a variety from the United States was used in this research purchased from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS), which is known to be susceptible to late blight. The potato was subcultured in MS medium with 6 g/L agarose and 30 g/L glucose; pH value was adjusted to 5.8. The leaf of the tissue cultured plant was used as the explant after grown at 3–4 weeks old.

3.2 hrap gene and its expression plasmid

The hrap gene was regulated by the CaMV35S promoter and its expression plasmid, pBI-HRAP was transformed into Agrobacterium, which was kindly provided by Prof. Teng-yung Feng from Institute of Botany, Academia Sinica in Taiwan. The plasmid structure is shown in Figure 5.

![Figure 5 The structure of plasmid harbouring HRAP in pBI121](image)

3.3 Genetic transformation

The explant from the tissue cultured potato leaflets were placed on CIM medium (medium formula: BAP 2.0 mg/L, NAA 0.2 mg/L, 2,4-D 0.2 mg/L, glucose 16 g/L, agarose 6 g/L and finally adjusted pH to 5.8), and pre-cultured for 2 days at 25°C in the dark. Then the explants were immersed for 20 min in the Agrobacterium inoculum kept an optical density of about 0.5 at 600 nm (OD600), and co-cultured for 2 days with Agrobacterium in the same medium and condition. Explants were transferred to GIM medium (CIM medium (30 g/L glucose) supplemented with 500 mg/L carbenicillin and 50 mg/L kanamycin (Kan, Sigma-Aldrich, St. Louis, MO, USA)). After about three weeks, the leaves with calli are transferred into SIM medium (GIM with BAP 2.0 mg/L, NAA 0.2 mg/L, 2,4-D 0.2 mg/L replaced by BAP 2.5 mg/L and GA 3 5.0 mg/L) to induce adventitious bud. Once the adventitious buds reached 2 to 3 cm in height, they were cut and transferred to CIM for subculture. The adventitious buds were then again removed as they reached a height of 2 to 3 cm and transferred to rooting medium (RM: MS medium supplemented with 75 mg/L Kan, 6 g/L agarose and 30 g/L glucose, pH 5.8). Regenerated plantlets rooted normally were
subcultured and others were transplanted into the nutrition pots. Except pre-culture and co-culture, all the cultivation conditions were 12000 LX light intensity, 12 h/12 h (light/dark) and temperature of (22±1)℃.

3.4 PCR detection
After four to five weeks of the regenerated transgenic plants growing in a pot, the lamina of different strains were collected for DNA extraction according to the method reported by Fulton et al (1995), followed by polymerase chain reaction (PCR) trace analysis. DNA was used as the template, with the plasmid pBIHRAP with the gene hrap as positive control (about 720 bp), and the non-transformed plant as negative control. The PCR system included 25 µL of reaction liquid containing 10×Buffer 2.5 µL, dNTPs (10 mmol/L) 0.5 µL, Taq polymerase (2.5 µmol/L) 0.5 µL (5’–GTTGGAGTTGGAGGACGAGG–3’), primer I (10 µmol/L) 0.5 µL (5’–CGCGGATCCATGAAAATGACGAC–3’), primer II (10 µmol/L) 0.5 µL (5’–GGGATCCCATGAAAATGAGAACCTCTC–3’), add ddH2O to 25 µL final volume. PCR was conducted under the following conditions: an initial step of 7 min at 94℃, followed by 30 cycles of 50 s at 94℃, 50 s at 53℃ and 60 s at 72℃, and a final extension step of 10 min at 72℃. The products were stored at 4℃.

3.5 Southern Blot analysis
The CTAB method was used to extract DNA (Doyle and Doyle, 1987), followed by restriction digestion of genomic DNA with BglIII (Promega Corp., Madison, WI, USA). After complete digestion, the sample was subjected to 1% agarose gel electrophoresis and transferred to a nylon membrane for Southern blot. Hybridization (with color) and probe preparation were performed following the instructions specified in the PCR DIG Labeling Mix (Roche Corp., Basel, Switzerland).

3.6 RT-PCR analysis
Extraction of total RNA: A leaf from each of 10 putatively-transformed potato plants from 10 independent transformation lines was ground into a fine powder in liquid nitrogen. Total RNA was isolated by using the TRizol® reagent Kit (Tiangen Biotech Co., Ltd, Beijing, China).

RNA retrovirus reaction system: Using RNA retrovirus Kit (1st Takara Strand cDNA bio-synthesis Kit) according to use instructions. Reaction conditions: 30℃ 10 min; 42℃ 60 min; 70℃ 15 min; 8℃ preservation.

cDNA template amplification hrap gene reaction system (25 µL): Taq PCR MasterMix 12.5 µL, forward and reverse primer each 1 µL, cDNA template 3 µL, ddH2O 7.5 µL. PCR was conducted under the following conditions: an initial step of 3 min at 95℃, followed by 34 cycles of 50 s at 94℃, 50 s at 53℃ and 60 s at 72℃, and a final extension step of 10 min at 72℃. The product was preserved at 8℃.

PCR products were subjected to electrophoresis in a 1.2% agarose gel and photo-documented under UV light. The Products were purified using the Tiangel Mini/Midi Purification kit (Tiangen), cloned into pMD18-T vector (Takara) and then transformed into DH5α-competent cells (Invitrogen, Carlsbad, CA, USA). Inserts from independent clones for each gene were sequenced at Beijing Sunbiotech Co., Ltd (Beijing, China). The sequence of hrap genes isolated from transformation plants were compared with hrap genes form GenBank.

3.7 Assay of disease resistance
3.7.1 Identification of the resistance of P. infestans
The purified test strains of P. infestans (ZY15, LSX18 and XH05-5-4) were cultured in rye-tomato juice media. After 10–15 days, the culture dish was covered with mycelia. A small volume of water was added into the dish and the surface of the mycelia was lightly scraped in order to make the sporangium fall into water. The solution was then filtered applying 270-mesh filter, and the number of sporangium in the filtrate was determined. The target concentration was 1000–2000 sporangium/mL of filtrate. The potato test-tube plantlets were grown in nursery pots, with 1–4 plants per pot at room temperature. The plants were inoculated when they had six to nine leaves. The inoculum of the sporangium suspension was sprayed on the potato plants, which were maintained in a humid environment for 24 h and monitored for the presence of disease.
3.7.2 Identification of bacterial wilt

Bacterial wilt strain TB48 was prepared on a triphenyltetrazolium chloride (TTC) plate, inoculated into TTC medium and cultivated at room temperature in a shaking incubator at 150 rpm for use. The prepared bacilli were added to a culture bottle containing MS medium (OD520=0.2). Different strains of potato plants were placed into the bottle, which was maintained in a greenhouse with daily observations.

Authors' contributions

JMH, LL and DYP carried out the trait of transformation, PCR and DNA extraction. CJ worked on the vector construction and Southern Blot analysis. ZZY worked on RT-PCR and DNA sequencing. LZP participated in the design of experiment and the modification of the manuscript. HRQ conceived the overall study, performed the experiment designs and took part in the data analysis as well as the preparation of the manuscript. All the authors have read and approved the final manuscript.

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