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Full Length Research Paper

In planta transformation of rice (Oryza sativa) using thaumatin-like protein gene for enhancing resistance to sheath blight

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Among the fungal diseases, sheath blight caused by *Rhizoctonia solani* Kuhn is one of the most important wide spread diseases found in all the rice plants (*Oryza sativa*) growing countries. The control of the disease with agricultural alternation and traditional breeding has not been successful so far. Application of fungicide is not recommended. Resistant genes which can cause immunity to the disease have not been found. D34 is a thaumatin-like protein (TLP) belonging to the group 5 of pathogen-related proteins (PRs). These proteins can probably change permeability of fungal membrane. In this research, an attempt was made to enhance the resistance to the sheath blight fungus on rice plant through the expression of TLP gene under the control of a CaMV35 promoter. To achieve this goal, rice plants (*O. sativa*) were transformed by the pAJ21-CaMV35S-tlpD34 construct via *in planta* method using *Agrobacterium tumefaciens* EHA101. The transformed plants were confirmed using polymerase chain reaction (PCR) amplifying a 710 bp fragment of the cloned gene.

Key words: *In planta*, pathogenesis-related protein, *Rhizoctonia solani*, rice, sheath blight, thaumatin-like protein, transformation.

INTRODUCTION

In plants, host disease resistance is an active phenomenon involving many biochemical components. Plant resistance mechanisms include the hypersensitive response (HR) and systemic acquired resistance (SAR) (Ross, 1961b). HR and SAR are involved in the up-regulation of the pathogen-related proteins (Bowles, 1990; Song and Goodman, 2001). On the basis of the amino acid sequences, serological relationship, and/or enzymatic or biological activity, there are 17 recognized groups of pathogen related (PR)-proteins, designated PR-1 through PR-17 in various plant species (Okushima et al., 2000; Christensen et al., 2002; Kostoff, 2005; Liu et al., 2010). Many PR-5 proteins have been found to have an antifungal activity (Koiwa et al., 1997, 1999; Wang and Ng, 2002; Van loon et al., 2006; O'Leary et al., 2007; Wang et al., 2010; Dhekney et al., 2010). The antifungal activity is presumably attributed to PR-5 induced membrane leakage and hyphal rupture of fungal pathogen (Robert and Selitrennikoff, 1990; Vigres et al., 1992; Daolin et al., 2005). Then, PR5 proteins generally exert their antifungal activity through a very fast and dramatic increase in the permeability of the pathogen's plasma membrane, by disrupting the lipid bi-layer and creating trans-membrane pores, though the exact molecular mechanism underlying the PR-5 antifungal activity is not known (Liu et al., 2010; Wang et al., 2010; El-Kereamy et al., 2011). PR-5 proteins may also indirectly contribute to other defense regulatory mechanisms such as phenylpropanoid and phytoalexin pathways (El-Kereamy et al., 2011).

Initially, a thaumatin-like protein (TLP) cDNA clone has been isolated and characterized from rice leaves infiltrated with *Pseudomonas syringae* pv *syringae*, a non–pathogen of rice (Reimmann and Dudler, 1993). In rice plants infected with *Rhizoctonia solani*, two TLPs, C22 and D34, have been identified which were entirely different from the TLP described previously (Velazhahan

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Abbreviations: **TLP**, Thaumatin-like protein; **PRs**, pathogen-related proteins; **PCR**, polymerase chain reaction.

et al., 1998). The rice TLPD34 was detected two to six days after inoculation, with a peak on the fourth day (Velazhahan et al., 1998). pGL2-CaMV35S-tlpD34, harboring TLPD34 gene was used to transform protoplasts of indica rice cultivars by polyethylene glycol (PEG)-mediated direct gene and immature embryos by biolistic transformation (Datta et al., 1999). A rice thaumatin-like protein gene (tlp-D34) and a rice chitinase gene (chi11) were introduced into the spring wheat cultivar 'Bobwhite' by co-transformation of the plasmids pGL2-CaMV35S-tlpD34 and pAHG11-CaMV35S-chi11. The transformation was by biolistic bombardment (Chen et al., 1999). A cDNA encoding TLP from rice was cloned into the binary vector pMON410 under the control of the CaMV35S promoter for *Agrobacterium*-mediated transformation of tomato (Radhajeyalakshmi et al., 2004). Transgenic rice which led to an enhanced resistance to sheath blight pGL2-ubi-tlpD34, harboring TLPD34 gene, and pMKU-RF2-chill, harboring chitinase gene, were used to transform mature and immature embryos of indicia rice cultivars by biolistic transformation (Kalpana et al., 2006). Furthermore, constitutive overexpression of TLPD34 in transgenic wheat plants by biolistic transformation delayed the onset of infection by *Fusarium graminearum* (Caroline et al., 2007). The purified rice TLP also inhibited the mycelial and germ tube growth of some other fungi *in vitro* (Jayaraj et al., 2004).

Since the breakthrough of Hiei and colleagues in the early 1990s, *Agrobacterium tumefaciens*-mediated transformation methods for rice have advanced and become the routine methods first inoculated onto immature embryos and calli, in a tissue culture system. However, they require sterile conditions and are time–consuming. Also, somatic mutation or somaclonal variation frequently occurs in plant cells during *in vitro* culture, especially in the monocotyledon plants, because of lack of special inducer and inability in entering to the nuclear and integration to plant genome, and some plants are recalcitrant to regeneration (Hiei and Koman, 2008). On the other hand, *in planta* transformation involves no *in vitro* culture and eliminates the chances of somaclonal variation induced through *in vitro* culture (Supartana et al., 2005). *In planta* transformation has been demonstrated in other species like *Arabidopsis thaliana*, buckwheat, kenaf, rice, cotton, *Brassica napus* and wheat previously (Feldmann and Marks, 1987; Kojima et al., 2000; Kojima et al., 2004; Supartana et al., 2005; Keshamma et al., 2008; Li et al., 2010 and Razzaq et al., 2010). In this report, we carried out some modification of this method to increase the efficiency of integration of the recombinant vector harboring the TLPD34 gene.

MATERIALS AND METHODS

Materials and instruments

Sodium hypochloride, tryptone, yeast extract, agar and salts for preparation of Agrobacterium induction media (AB), Luria-Bertani (LB), super optimal broth (SOB), yeast extract peptone (YEP), super optimal broth with catabolite repression (SOC), Yoshida and other solutions were all from Merck (Frankfurt, Germany). *Escherichia coli* JM101, pAJ21, all the enzymes and various size markers were prepared from Fermentas (St. Leon-Rot, Germany). Ampicillin, rifampicin and cefotaxime were prepared from CoAlhavi (Tehran, Iran). Primers were purchased from GeneWorkCo (Thebarton, Australia). dNTPs were from Roche (Mannheim, Germany) and other reagents used for polymerase chain reaction (PCR) were from Sinagen (Tehran, Iran). 2-[4-morpholino]-ethane sulfonic acid (MES) for preparation of induction medium and acetosyringone were from Sigma (Deisenhofen, Germany). Rice (*Oryza sativa* var Hashemi) seeds were obtained from Rice Research Institute (Rasht, Iran).

Instrument used in this investigation were as follows: gradient thermal cycler for polymerase chain reaction; spectra photometer (Eppendorf) for quantification of nucleic acid; electroporation for transformation of bacteria; gel documentation system for image acquisitions. Full-length cDNA preparation for TLPD34 gene pFLCI harboring full-length cDNA of TLPD34 gene, sequence producing significant alignment, was purchased from NIAS (Ibaraki, Japan).

Introduction pFLCI to *E. coli*

Rice pFLCI harboring TLP gene was delivered to the *E. coli* JM101 by electroporation, after preparation of *E. coli* competent cell by calcium chloride procedure. The LB medium supplemented with ampicillin sulfate (50 µg/ml) was used for selection of the transformed colonies.

Extraction of plasmid DNA for PCR analysis

Plasmid extraction of *E. coli* JM101 with pFLCI plasmid containing TLPD34 gene and *A. tumefaciens* EHA101 with pAJ21 plasmid containing TLPD34 gene was carried out according to the Quick gel QIA extraction kit instruction.

Construction of TLPD34 gene delivery vector

A 1.1 kb DNA fragment was obtained by digestion of pFLCI with *Bam*HI and *Xho*I. Digestion of pAJ21, a binary vector with CaMV35S promoter fragment excised for *A. tumefaciens* over expression with the same enzymes was carried too. pAJ21-CaMV35S and the 1.1 kb *Bam*HI-*Xho*I fragment of the TLPD34 gene were mixed together and ligated to yield pAJ21-CaMV35S-tlpD34 by Fermentas protocol. pAJ21-CaMV35S-tlpD34 construct was delivered to the *E. coli* JM101 by electroporation method. Transformed colonies were selected in LB medium. After plasmid extraction, sequence analysis was carried out (Macrogene, Tokyo, Japan).

Introduction of construct to the *A. tumefaciens*

Introduction of construct to the *A. tumefaciens* EHA101 was carried out by virtue of electroporation method, and then the transformed colonies were spread on LB medium containing rifampicin sulfate (75 µg/ml) and ampicillin (50 µg/ml). This approach led to an appropriate primary selection of *Agrobacterium* strain harboring pAJ21-CaMV35S-tlpD34. Several selected colonies were subjected to PCR based on the amplification of TLPD34 gene (710 bp) using TLP primers.

PCR test

PCR was used for amplification of a 710 bp of the full-length cDNA

of tlpD34 using specific primers: tlp forward (5′-ACCAAAGCCATGGCGCCTTCCCT-3′) and tlp reverse (5′-TTCGATCATGGGCAGAAGACGAC-3′).

In planta transformation

We carried out the transformation by the Gelvin method (2006). *A. tumefaciens* was cultured on LB medium containing rifampicin (75 µg/ml) and ampicillin (50 µg/ml) at 28°C for 16 h. Afterward, 0.5 ml bacterial suspension was cultured on AB medium containing glucose (0.5%) at 28°C and 230 rpm for 16 h. Bacterial plate at OD_{600} was assembled and cultured on inducing medium contained acetosyringone (100 µM) at 25°C and 50 rpm for 14 to 24 h. Bacterial plate was solved in 1.2 Murashige and Skoog (MS) medium. Rice (*O. sativa* var Hashemi) seeds were sterilized by soaking in 90% ethanol (1 min) and washed with water three times. Sterile seeds were placed on wet cotton at 22°C for two days. At this stage, *A. tumefaciens* was inoculated into embryonic apical meristem of the soaked seeds, a region on the seed surface where a shoot would later emerge and was pierced twice up to depth of about 1 to 1.5 mm with a needle (Φ 0.70 mm) dipped in the *A. tumefaciens* inoculums. The inoculated seeds were then placed on filter papers on wet perlite in flasks covered with aluminum foil and incubated at 23°C in dark for nine days, during which 70 to 75% of inoculated seeds germinated to seedlings. In order to eliminate *A. tumefaciens*, the seedlings were immersed, at room temperature, in an aqueous solution (1000 ppm) of cefotaxime for 1 h. Subsequently, seedlings were transformed to basins containing Yoshida solution for rooting. Finally, seedlings were planted in pots and grown to maturation (T_0) under non-sterile conditions and allowed to pollinate naturally to set seeds (T_1).

Inoculation of rice plants by *R. solani*

The T_0 and T_1 generation of transformants were infected with *R. solani*, 40 days after planting. All the *R. solani* inoculations were carried out with mycelial disc of 5 mm diameter, obtained from three-day-old culture of *R. solani* grown on potato dextrose agar at 28°C. Each selected leaf sheath was inoculated with a single mycelial disc. The mycelial disc placed on leaf sheath, was covered with absorbent cotton and secured with parafilm. The cotton was moistened periodically with sterile distilled water, to maintain high humidity (Anuratha et al., 1996).

Extraction of genomic DNA from leaves

Genomic DNA for PCR was isolated from young leaves at the shoot apices of rice plants of transformed plants according to cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990).

RESULTS AND DISCUSSION

Full-length cDNA (clone ID: 301152 and 98% homology with TLPD34 gene) was selected in KOME site. PCR with specific primers for amplification of a 710 bp of full-length cDNA was used as a method for the confirmation of existing full-length cDNA in pFLCI. Cloning of pFLCI in *E. coli* JM101 was performed for more reproduction for further uses. As shown in Figure 1, cloning was confirmed by PCR reaction and results showed that all of the selective colonies had a 710 bp band in gel electrophoresis. The result (710 bp) was subjected to series of excision and sub-cloning processes towards production of pAJ21-CaMV35S-tlpD34 construct. The insertion of TLPD34 gene in pAJ21 showed a 6000 bp band in gel electrophoresis (Figure 2). For selection of transformed *A. tumefaciens*, very low amount of the bacteria was used to ensure the colony selection process confirmed by PCR (Figure 3) and insertion of TLPD34 gene in pAJ21 confirmed by sequence analysis too (data not shown).

Regeneration of inoculated seeds

The seeds were sterilized and inoculated. Regeneration percentage was 59% (Table 1). Piercing the seeds might have damaged mother line germ cells. Therefore, 39% treated seeds could not regenerate into seedling. Piercing meristematic cells with needles is very critical for the determination of inoculating mother line germ cells and attention should be taken to avoid the mother line cells from damage. It is necessary bacteria infect mother line cells. The embryo was visible when inoculated and choosing the best position of embryo for inoculation was possible.

Induction of thaumatin-like protein in *R. solani*-infected rice plants

Rice plants were infected with *R. solani* at maximum tillering stage. In transgenic lines, the disease lesions were relatively smaller and surrounded by a conspicuous defensive browning, while in the non-transgenic controls, the disease lesions were larger (Figure 4), leading to morbid yellowing of leaves. Moreover, in the leaf sheaths of transgenic line, the spread of lesion was slower and the lesions were surrounded by zone of extensive browning. However, in non-transformed controls, blanched lesion appeared, the faster spread of which led to complete drying of infected leaf sheaths.

Molecular evolution of putative transformants

Transformed status of the putative transformants was evaluated by the presence of TLPD34 transgene in the genomic DNA by PCR analysis. PCR analysis for some samples is presented in Figure 5. PCR results showed that 24% plants inoculated with *Agrobacterium* integrated transgene (Table 1). *In planta* transformed rice plants have been obtained recently by Supartana et al. (2005). Therefore, TLP gene can be delivered successfully into growing meristem cells of rice as is evident from this experiment. The transformed plants were confirmed using PCR amplifying a 710 bp fragment of TLPD34 gene.

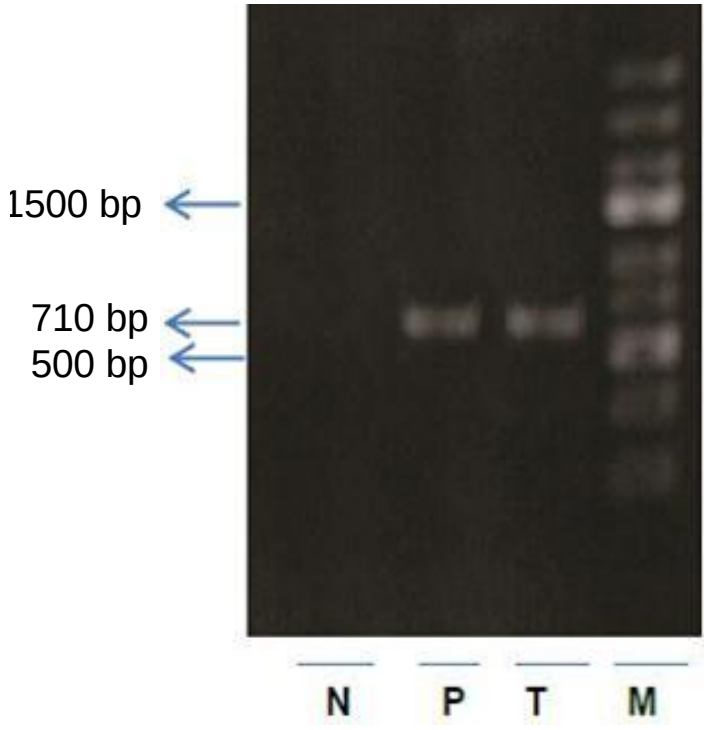

Figure 1. PCR product of the cloned full-length cDNA of TLP gene on the 1% agarose gel. PCR for confirmation of *E. coli* transformation, M, 100 bp marker; T, amplified TLPD34 gene; P, positive control; N, negative control. PCR, Polymerase chain reaction.

Figure 2. pAJ21-CaMV35-tlp on 1% agarose gel. M, 1 k bp marker; lane 1, pAJ21 before insertion of TLPD34 gene; lane 2, pAJ21 after insertion of tlpD34.

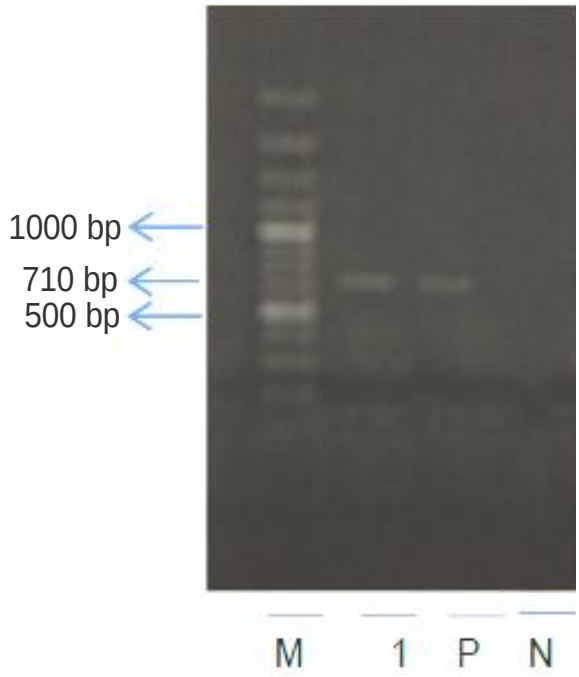

Figure 3. PCR product of transformed *A. tumefaciens*. M, 100 bp marker; lane 1, colony with a 710 bp band; P, positive control; N, negative control. PCR, Polymerase chain reaction.

Table 1. Percentage of PCR positive plants after inoculation of apical meristem with *Agrobacterium*.

Detail of material	Inoculation
No. of treated seeds	800
No. of inoculated seeds	550
No. of regenerated seeds	327
Regeneration percentage	59%*
Number of PCR positive	80
Percentage of PCR positive plants	24%**

Percentage calculated based on number of seeds inoculated; **percentage calculated based on number of regenerated seeds.

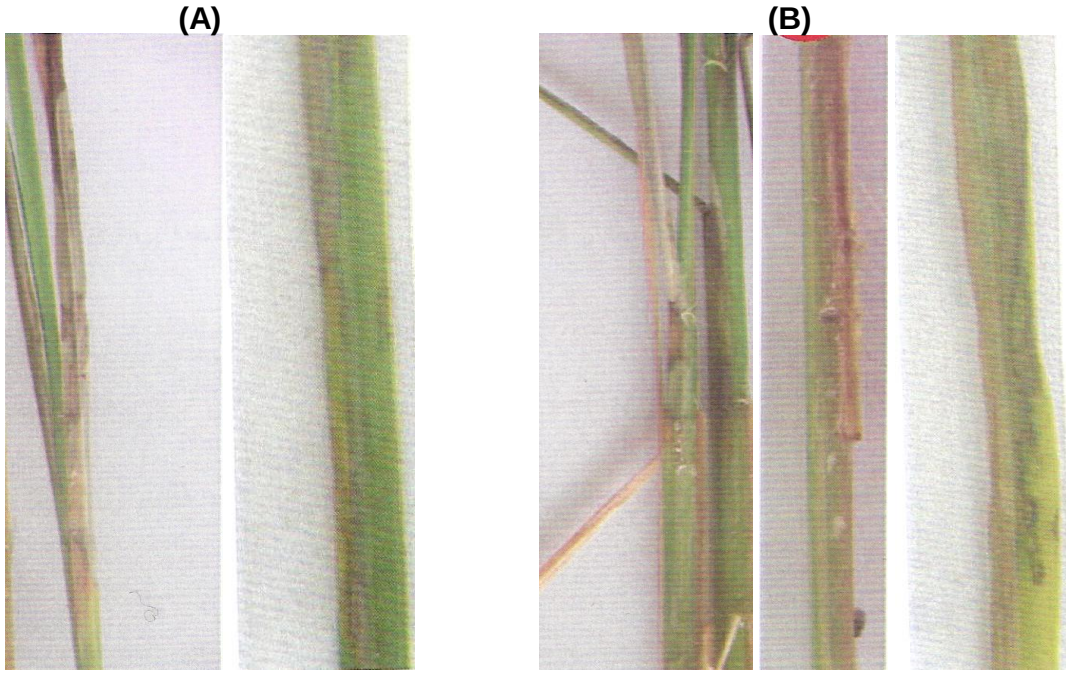

Figure 4. Assessment of sheath blight development in transformed rice plants expressing TLP (A) and non-transformed rice plants (B). TLP, Thaumatin-like protein.

Sequencing analysis of putative transformants

A search of DNA sequence data base in National Center for Biotechnology Information (NCBI) revealed that sequenced DNA shared sequence similarity to the TLP34 gene deposited with Genebank under accession number U77657 by Velazhahan et al. (1998) (Figure 7). The results of this experiment reveal the success of delivery and stable integration of foreign gene into meristem cells of rice. The results also provide a positive evidence for success of *in planta*. This indicates a good efficiency of this method compared with particle bombardment and *Agrobacterium*-mediated transformation involving tissue culture. This prevents essential tissue culture requirement that needs a good skill, expense and labor to produce handful of transformed plants. Moreover, this method removes the probabilities of somaclonal variation induced through *in vitro* culture. *In planta* method is a potentially useful approach for cereals where regeneration of transformed plantlets is insufficient. *In planta* developed for transformation of rice through apical meristem can renew the genetic progress of it to cope with its fast increasing demand and has the potential for effective genetic transformation of other crops through growing meristems.

In the present study, we were able to produce transgenic lines of Iranian rice cultivar by *in planta* method and inheritance of TLP gene was confirmed into the genome of T_1 plants. Until now, considerable efforts have been made for optimization of *Agrobacterium*-mediated

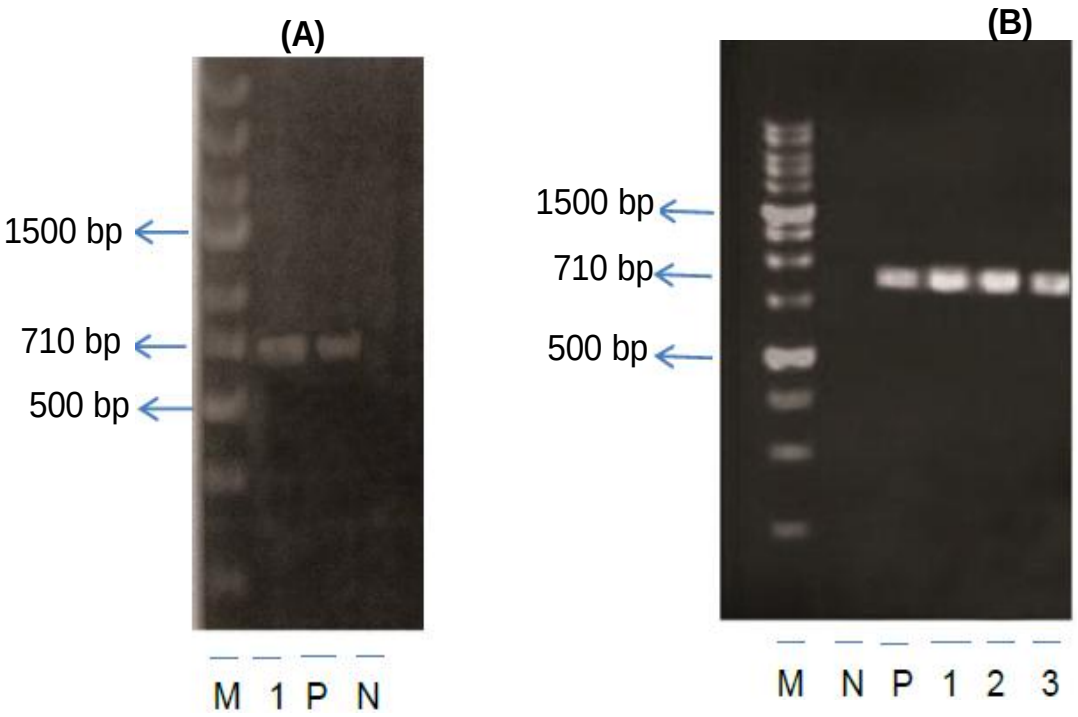

Figure 5. Detection of transgene in transformants. A) PCR reaction of T_0 transformants. M, 100 bp marker; lane 1, PCR product of T_0 transformants. B) PCR of T_1 transformants. M, 100 bp marker, lanes 1 to 3, PCR products of T_1 transformants; P, positive control; N, negative control.

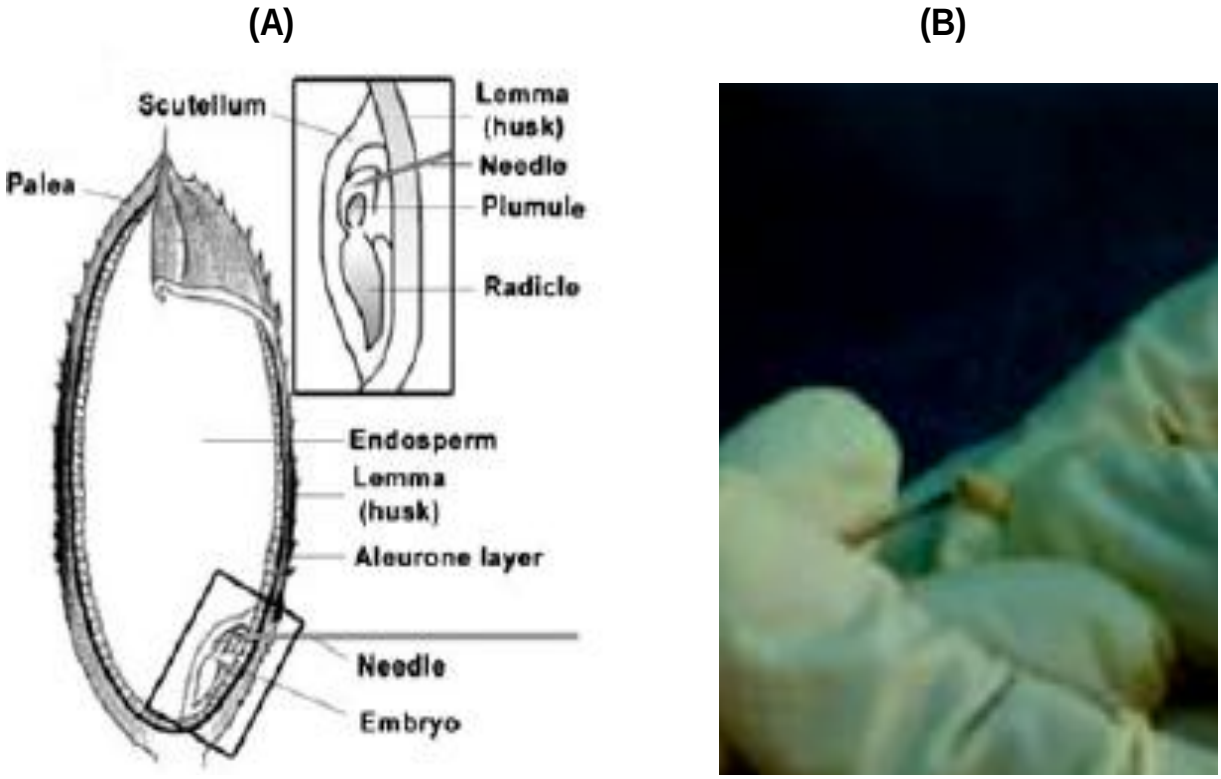

Figure 6. The position of inoculation of rice seeds. A) The position of plumule and embryonic apical meristem (Lin et al., 2009). B) The side of apical meristem of soaked seeds inoculated by *A. tumefaciens.*

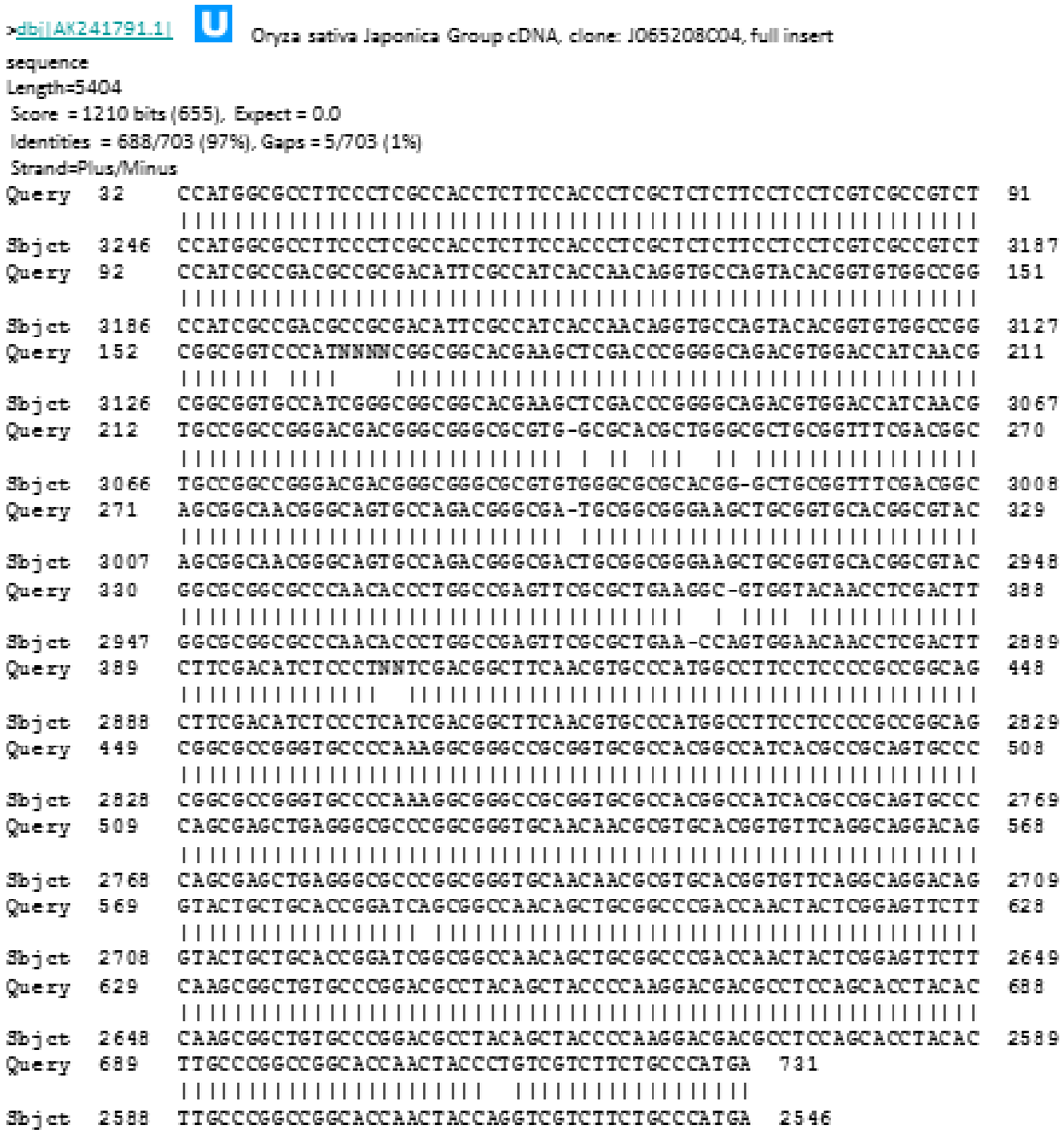

Figure 7. BLAST of the sequence of insertion fragment in T_1 plants in NCBI. NCBI, National Center for Biotechnology Information.

transformation of mature embryos and development of direct *Agrobacterium*-mediated transformation of germ-line cells in seeds, shoot meristem and immature embryos (Dai et al., 2001; Urushibara et al., 2001; Hiei and Koman, 2008; Azria and Bhalla, 2011). Modification of different genetic and environmental aspects of transformation method may lead to better understanding of the system and result in high efficiency (Alimohammadi and Bagherieh-Najjar, 2009). Efforts have also been devoted to deliver exogenous DNA to the cells of cereals meristems via electrophoresis, p article bombardment and microinjection (Razzaq et al., 2011).

Meristem cells of cereals can be targeted successfully for delivery of DNA (Iglesias, 1994) and are competent

for stable transformation (Potrykus, 1990). Therefore, imagining the importance of tissue culture free genetic transformation, a study was done for *in planta* apical meristem transformation of rice through *Agrobacterium* to study an easy, cheap and efficient method. *A. tumefaciens* was inoculated on to the embryonic apical meristems of soaked seeds, in which various organs and primordial had already been formed. Conceivably, *A. tumefaciens* could transfer its T-DNA not only to the cells of apical meristems, but also to the cells of various organs and primordia of the soaked seeds. Consequently, it is likely that transformants at the T_0 stage were chimeras and at the T_1, *A. tumefaciens* transfer was only to the genomes of the cells. The best position for inoculation is side of apical meristem of soaked seeds in which plumule will form later (Chang and Bardenase, 1965). Plumule may be destroyed later, if needle pierce into it directly (Lin et al., 2009; Figure 6). To put it differently, *in planta* transformation method mimicked the infection process of *A. tumefaciens* of plants in nature. This might leads to the high transformation efficiency of *in planta* method.

We used acetosyringone (100 µL), *A. tumefaciens* EHA101 and glucose, as another inducing substance; according to optimal protocol, introduced acetosyringone enhances expression of *vir* genes (Hiei and Koman, 2008). Acetosyringone was included in this experiment as an essential factor required for the transcriptional activation of *Agrobacterium* virulence machinery (Seo et al., 2002; He et al., 2010). Nevertheless, some of the researchers think this is not an absolute requirement (Bartlett et al., 2008). It seems likely that the other inducing substance, monosaccharide, plant hormone, vacuum and other strains of *Agrobacterium* can increase the efficiency of *in planta* method. Of importance is that their effectiveness can be probed in the molecular level and transcription of inducer gene like *virG* and *virD*. TLP transgenic lines produced in this study could be used as a source of resistance to other fungal diseases.

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