CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Whisker-mediated transformation of peanut with chitinase gene enhances resistance to leaf spot disease

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Abstract: *Peanut (Arachis hypogaea) is an important legume and oilseed crop, native to South America and grown in all tropical and temperate regions of the world. A simplified and rapid direct gene delivery system in peanut was developed by vortexing silicon carbide whiskers with callus and with plasmid harboring chitinase and hygromcin genes. The effects of callus age and whisker quantity on transformation efficiency were evaluated. Transformation efficiency (6.88%) was highest when 200 mg of whiskers were used with 5 µg plasmid for 2 g of 20-day-old callus. Hygromcin-resistant calli were regenerated to complete plants which produced seeds normally. Transgene insertion and number of transgene copieswere confirmed by PCR and southern blot analyses, respectively. Transgene expression was evaluated by a pathogenecity test and RT-PCR analysis. In transgenic events, the resistance level to leaf spot disease was far higher than in control plants.*

Key words: *Transgenic, callus, reporter gene, cell wall.*

INTRODUCTION

Because of the narrow genetic base, improvement in peanut by conventional breeding is not as rapid as needed to meet the demands of a growing population (Asif et al. 2011, Banjara et al. 2012). Genetic transformation is the most appropriate option for breeding of many important traits that are not improved by conventional methods. The establishment of a suitable gene delivery system and a protocol for the subsequent recovery of plants is a basic requirement for the recalcitrant varietiesof peanut (Branch 1996).

The plant cell wall is frequentlyaninanimate obstacle in experiments with DNA delivery technologies for plant genetic engineering. In case of biological systems, the cell wall is dissolved by enzymes, secreted by mediating prokaryotes, which allows DNA delivery to recipient cells (Dunwell 2011). The cell wall problem was circumvented by usingprotoplasts for DNA bombardment in non-*agrobacterium* transformation methods. Protoplasts are excellent targets for genetic transformation, whereas plant regeneration from protoplasts is problematic (Choi et al. 1997). Genegun-mediated transformation is also receiving increasing attention, but requires sophisticated expertise, expensive equipment and consumables. Silicon carbide whiskers on the other hand are extremely well-suited means of direct gene delivery, for being very simple and

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not requiring much technical equipment and skills (Kaeppler et al. 1990). These whiskers are basically 10-80 mm long and 0.6 mm widemicrofibers (Coffee and Dunwell 1994).

Silicon carbide whiskers are inherently hard and easily cleavable, producing sharp cutting edges (Greenwood and Earnshaw 1984). The mechanism for whisker-mediated transformation is based on the micro puncturing of cells for DNA delivery. The surface of silicon carbide whiskers is negatively charged so it cannot bind to DNA for ultimate delivery into the host cell (Appel et al. 1988, Mutsuddy 1990). Kaeppler et al. (1992) also refuted the benefit of premixing DNA with whiskers. This means that whiskers puncture the cell only during vortexing, opening the way for DNA entry. However, to develop a protocol for consistent transgenic plant production, some marker genes were used (Dalton et al. 1998). In this sense, the conditions for a successful whisker-mediated DNA transformation have been studied using the transient expression of reporter genes (Nagatani et al. 1997, Iqbal et al. 2012). Moreover, for the whisker method, tissue culture with specific morphological features has to be produced and maintained, as well as conditions that favor an efficient delivery of DNA to target cells (Petolino 2002). In addition, an effective means of isolation is a pre-requisite for the recovery of infrequent integration events (Thompson et al. 1997).

When peanut is exposed to fungal attack, the plants accumulate pathogenesis-related proteins such as chitinases (Brunner et al. 1998), indicating antifungal activity (Yun et al. 1996). In tobacco plants, on the other hand, different chitinase isoforms were identified bystructural gene analysis, although only particular isoforms exhibited antifungal activity (Sela-Buurlage 1993, Chu et al. 2013, Parasad et al. 2013).

The purpose of this study was to develop a rapid, simple, economical and less laborious protocol for a highly efficient silicon carbide whisker-mediated transformation of a peanut cultivar.

MATERIAL AND METHODS

Production of embryogenic callus

The epicotyl explants of four commercial peanut varieties (BARD-92, BARI-2000, BARD-479, and Golden) were cultured on callus induction medium (CIM) consisting of MS (Murashige and Skoog 1962), macro- and micro-salts, B5 vitamins (Gamborg et al. 1968), and 30g L^{-1} sucrose, and supplemented with 8 mg L^{-1} picloram, as previously standardized by Hassan et al. (2013). Thereafter, 8g L^{-1} agar was added after adjusting the pH to 5.8 at 25 ^{0}C. The explants were inoculated on medium for 3 weeks at 25± 2 ^{0}C, with a photoperiod of 16/8 hours. The medium, growth regulators and agar were supplied by Phytotech Lab, USA.

Preparation of silicon carbide whiskers and DNA delivery

Different quantities of silicon carbide whiskers (100, 200 and 300 mg) were added to pre-weighed 50-mL plastic tubes, covered with aluminum foil and autoclaved at 121 ^{0}C for 15 min. Five ml of separately autoclaved callus induction medium and 5 µg of the binary vector plasmid (pB1333) harboring rice chitinase and hygromycin phosphotransferase (hpt) genes driven by EN4 and CaMV 35S promoters, respectively, were added to this tube and vortexed for 2 min. Two gram of embryogenic callus (10, 20, and 30 days old) was added to this tube and vortexed again for 2 min and incubated on CIM for 24 hours at 25 ^{0}C. The control experiment was performed without plasmid DNA.

Selection for stable transformants

Treated callus clumps were transferred to petri plates containing callus induction medium with 30mg L^{-1} hygromycin (lethal dose). Surviving calli were sub-cultured on fresh medium after 2 weeks. The hygromycin-resistant calli were transferred to embryo conversion medium (ECM) consisting of MS salts, B5 vitamins, 30g L^{-1} sucrose and 1.5 mg L^{-1} 2,4-D, as standardized by Hassan et al. (2013). The developed embryos were transferred to embryo germination medium (EGM) consisting of MS salts, B5 vitamins, 30 g L^{-1} sucrosesupplemented with 0.1 mg BAP, as determined by Hassan et al. (2013). After 3 weeks, the germinated embryos were planted in coconut husk compost.

PCR Analysis

The DNA from putative (T_0) and control peanut plants was isolated by the CTAB method. For PCR analysis of chitinase

in the genome of transformants, forward primer 5'-CATATCAAGCATGAGGTGTA-3' and reverse primer 5'-CAACAACGATTTT GCTATAA-3' were used to amplify a 696 bp fragment. DNA was first denatured at 94 ^{0}C for 3 min and then for 30 s in each cycle. Annealing temperature was 52 ^{0}C for 30 s, while extension was run for 45 s at 72 ^{0}C. After 40 cycles, a final extension step was added at 72 ^{0}C for 20 min.

Selection of T_1 Plants

Lateral twigs of 25-day-old T_1 plants were cut, and thetrimmed end was submerged in Hoagland solution containing 150 mg L^{-1} hygromycin in test tubes. Plants with symptoms of discoloration, necrosis and wilting were discarded, while those with normal green color and vigor were selected and planted inlarger (25 x 25 cm) earthen pots.

Pathogenicity test for intact plants

The T_1 plants from which twigs were cut for the above test were tested for pathogenicity. Spores of *Cercosporadium personatum* were extracted by thoroughly washing a large number of infected leaves and collecting the spores from the distilled autoclaved water. Spore concentration was measured by a haemocytometer and adjusted to a concentration of 2 x 10^5 spores mL^{-1}. The resistance of control and transgenic plants was evaluated by spraying spore suspension on the leaves until run-off. An air humidity of 100% was maintained with a humidifier in the green house. The necrotic area (%) was calculated by multiplying the mean lesion size by the number of lesions divided by the leaf area.

RT-PCR analysis

The RNA from control and confirmed transgenic (T_1) plants was extracted with TRI reagent® (Sigma-Aldrich). The cDNA was synthesized using a Omniscript® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The total reaction volume of 20 µL contained 2 µg RNA, 2 µL 10x RT buffer, 2 µL dNTP Mix, 2 µL oligo-dT primer (10 µM), 1 µL RNase inhibitor (10 units μL^{-1}), and 1 µL reverse transcriptase. Polymerase chain reaction was performed using the primers mentioned in the section PCR analysis, and the product was separated on 1% agarose gel.

RESULTS AND DISCUSSION

Analysis of variance showed that callus age, whisker quantity and their interactions had highly significant effects on transformation efficiency (Table 1). The transformation efficiency was highest (6.88%) when 200 mg of whiskers were used for 20-day-old callus and lowest (2.00%) when using 100 mg of whiskers on 30-day-old callus (Figure 1). The turgidity of 20-day-old callus cells seems to be optimal, facilitating whisker piercing to deliver the desired foreign DNA (Asad et al. 2008).

Callus cells at the age of 10 days are fully turgid and thus more prone to excessive damage by whisker piercing (Petolino et al. 2000). On the other hand, 30-day-old callus cells lose much of their turgidity and are least wounded by whiskers, decreasing the transformation efficiency.

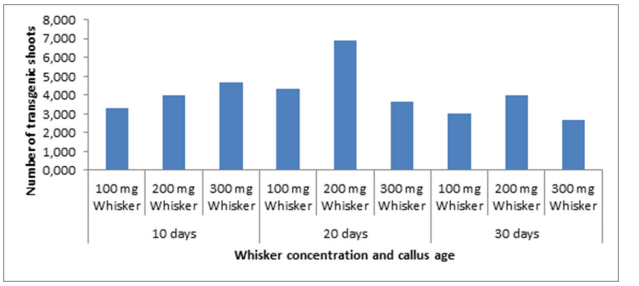

Figure 1. Effect of callus age and whisker concentration on numberof hygromycin-resistant shoots (%).

Table 1. Analysis of variance for factors affecting transformation efficiency

Source of variation	df	Sum of Squares	Mean Square	F Value
Callus age	2	15.50	7.75	20.92**
Whisker quantity	2	10.66	5.33	14.40**
Interaction	4	11.66	2.91	7.87**
Error	18	6.66	0.37	35.41
Total	26	44.50	3.02	34.42

** Highly significant

T_0 generation

Embryos with normal growth in selective medium (Figure 2) were subcultured on the same medium to confirm their resistance to hygromycin. Only three hygromycin-resistant embryos were successfully converted to full-fledged plants in which the expected 696 bp band was confirmed by PCR analysis (Figure 3). These plants were rooted in coconut husk compost, where they reached maturity and produced seeds normally. Southern blot analysis indicated a single copy of the gene only in plant number T_0-3, while two copies were observed in the other two plants (Figure 4).

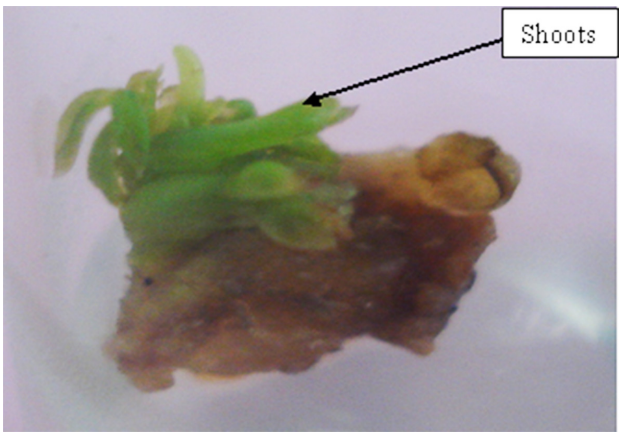

Figure 2. Emergence of hygromcine resistant shoots in selection medium.

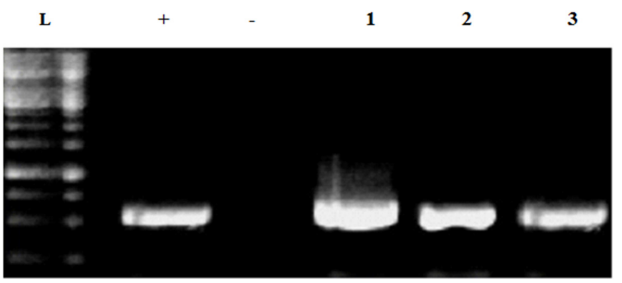

Figure 3. Amplification of RCG 3 gene fragment by PCR in three surviving T_0 plants. L: 1 kb ladder; (+): Plasmid containing RCG3 gene; (-): control plant; Lanes 1-3: transgenic plants (T_0-1 to T_0-3).

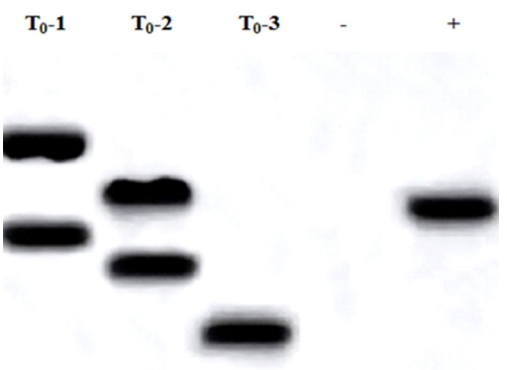

Figure 4. Southern blot analysis of three PCR positive plants.

T_1 generation

Among the progeny plants of T_0-3, six were resistant and five sensitive to hygromycin. Polymerase chain reaction analysis for the presence of gene RCG-3 revealed the expected 696 bp band in the hygromycin-resistant plants, while no band was observed in the sensitive plants (Figure 5).

Pathogenicity test

The uppermost leaf node, with four leaflets, was tagged on three main branches per plant for disease evaluation (Figure 6). The incubation frequency (IF, number of lesions per cm^2 of leaf area), incubation period (IP), lesion diameter (LD), leaf area damage (LAD), and disease score

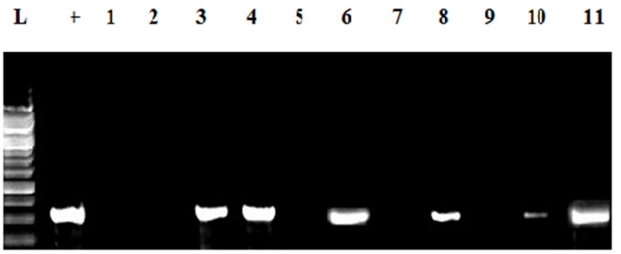

Figure 5. Amplification of RCG 3 gene fragment by PCR in T_1 plants. L: 1 kb ladder; (+): Plasmid containing RCG3 gene; Lanes 1-11: T1 plants.

Figure 6. Pathogenicity test of intact plants for susceptibility to late leaf spot disease: control (above) and transgenic (below) plants.

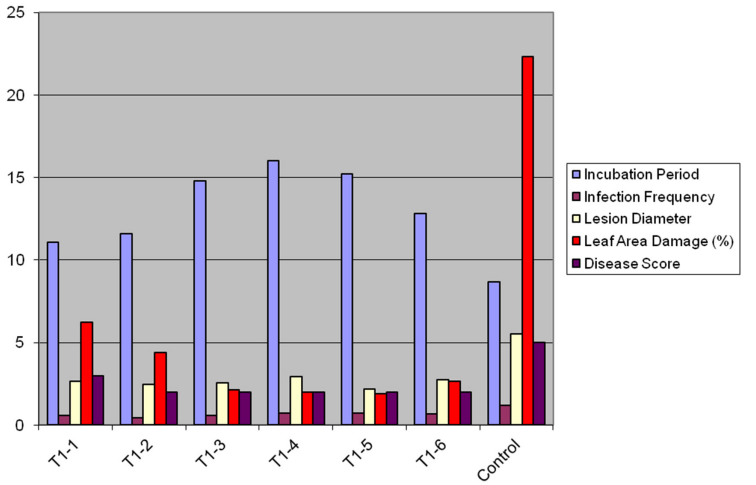

Figure 7. Means ofinfection frequency, incubation period, lesion diameter, leaf area damage, and disease score of transgenic and control plants.

were significantly higher in the control than in transgenic plants (Figure 7). This indicates the practical importance of transgenic plants to control leaf spot disease.

RT-PCR analysis

The reverse transcription polymerase chain reaction of 6 PCR-positive T_1 plants of the progeny plants of T_0-3 detected bands in all plants, although the band brightness and thickness differed significantly. In the plantslabeledT1-1, T_1-3 and T_1-6, the bands were much brighter and thicker, indicating a higher number of mRNA transcripts, which in turn indicates higher chitinase expression (Figure 8). The control plants did not have this band, while in the plants labeled T_1-2, T_1-4 and T_1-5, the band was comparatively less bright, indicating less mRNA copies, i.e., a reduced chitinase expression.

Silicon carbide whisker-mediated transformation is probably the simplest known method of genetic transformation. It involves vortexing of target tissues together with plasmid (harboring the gene of interest) in presence of silicon carbide. Silicon carbide is micro-needle shaped, one of the hardest known substances, and is used as an abrasive and as a component of saw blades (Dunwell 2011). Studies reveal that silicon carbide whiskers have a high tensile strength, elasticity and resistance to degradation (Choi et al. 1997). In addition, their intrinsic strength is high and they are easily cleavable, producing sharp cutting edges, as needed to penetrate cell walls and ultimately the cell nucleus (Greenwood and Earnshaw 1984). Electron microscope scanning shows that whiskers penetrate into maize cell nuclei, perforating the cell and opening the way for DNA entry (Kaeppler et al. 1990). Whisker-mediated cell perforation facilitates DNA movement to the nucleus. A higher whisker quantity and prolonged shaking can increase the transformation efficiency, whereas cell survival and regeneration are adversely affected. In other words, the whisker quantity, mixing speed and survival rate have to be well-balanced to optimize the efficiency (Frame et al. 1994, Petolino et al. 2000, Mizuno et al. 2004, Keshavareddy et al. 2013). Moreover, callus age affected the transformation efficiency greatly in different crops. In *Agrostis alba*, best results were obtained when transformation was performed after 6 days of subculturing (Asano et al. 1991), while for cotton, 14 days were optimal (Asad et al. 2008). In two experiments for silicon carbide-mediated transformation of rice, Matsushita et al. (1999) firstly vortexed scutellum tissues of embryos together with pAct1-F plasmid harboring the GUS gene and silicon carbide in liquid medium. They observed 302 GUS spots in a 250mg sample. In a second experiment, they used two plasmids; pAct1-F harboring the GUS gene and pDM302 the bar gene

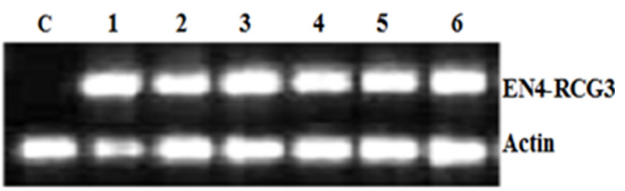

Figure 8. Six PCR-positive plants from progeny of T_0-3 plant were subjected to RT-PCR analysis to obtain mRNA transcripts.

(which confers bialaphos resistance). In the 873 embryos used in the experiment, bialaphos resistance was evidenced in 57 calli, some of which were also GUS-positive.

No report of any previous study on transformation of peanut mediated by silicon carbide whiskers was found in the literature for a comparison with this paper. The results of this study show that silicon carbide whisker-mediated transformation is a time saving and cost-effective system of insertion of a single transgene copy into legumes, particularly of peanut, without any problem of overexpression and inheritance. Moreover, the efficiency of transformation with the rice chitinase gene to control leaf spot disease in peanut was also evidenced.

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