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Improved Protocol for Agrobacterium-Mediated Transformation of Pea (*Pisum sativum*)

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Abstract

Pea is one of the most important legume crops whose production is constantly threatened by field and storage pests and diseases. Developing insect resistant transgenic pea plants through Agrobacterium-mediated transformation is a promising solution to maintain crop yield. However, the transformation efficiency is still low. Therefore, there was an attempt to enhance transformation efficiency by optimizing infection time, co-cultivation period and *in vitro* regeneration system. Transformation was performed using segments of embryonic axes from mature pea seeds (*Pisum sativum* L. cv. *Sponsor*). The segments were inoculated with the hyper virulent EHA105 strain of *Agrobacterium tumefaciens*. The Agrobacterium strain harboured a binary vector pGII35S containing the bar gene which confers resistance to phosphinotricin. The selection medium contained P2 medium with increasing concentrations of phosphinothricin. To improve the regeneration efficiency, 4.5 µM zeatin was added to the selection medium. The highest transformation efficiency (7.89%) was achieved with infection time of 90 min and co-cultivation period of 2 days. The shoots elongated well and the number of shoots/explants was increased (6 folds) after addition of zeatin. Resistant shoots were grafted onto rootstocks in soil and grafting success rate was 100%. The integration of *cry1Ac* gene in T_0 transgenic plants was confirmed primarily by Polymerase Chain Reaction and further analysed by Southern blotting.

Keywords: Pea; Agrobacterium; Co-cultivation period; Inoculation time; Transformation; Zeatin

Introduction

Pulses, including dried beans and peas, are cheap protein sources which contain essential micronutrients. Growing pulses contributes significantly to sustainable food security and prevent malnutrition, particularly in Latin America, Africa and Asia [1]. Among pulse crops, field pea (*Pea sativum* L.) is the third most important crop worldwide after common bean (*Phaseolus vulgaris*) and chickpeas (*Cicer arietinum*) [2]. The field pea contains about 23-25% protein which is used in combination with canola meal as a livestock feed [3]. Pea production is constrained by biotic and abiotic stresses. Insects and other pests annually prompt substantial loss of seed yield and quality. Pea weevil (*Bruchus pisorum* L.) is known to be one of the most devastating insect pests [4] which reduces yields up to 30% under heavy infestations [5]. Weevil-infested seeds have low germination rates and cannot provide stable crop yields [6]. Pea farmers often rely on pesticides as the only efficient means of control. However, this practise often is hampered by wrong application time to coincide with the female egg laying stage. Additional treatments may be required if weevil invasions continue for 2 to 4 weeks [7]. On the other hand, some of these chemical compounds endanger the natural microflora and fauna dwelling in the soil which play a key role in protecting plants against secondary infestation. These compounds can also develop resistance in the pests [8]. Recently, insecticide resistance in the pea and bean weevil has been observed in the United Kingdom where pyrethroid sprays have been used [9].

Using pod and seed resistant cultivars to *B. pisorum* would reduce control costs and provide a sustainable solution [10]. But breeding of legume crops faces number of challenges to develop storage insect resistance due to methodological limitations, biotypic variation, undesirable genetic linkages and limited knowledge of genetic bases of resistance [11]. In fact, using breeding methods to achieve the desired results are time-consuming. Therefore, genetic transformation is used to increase crop productivity by introducing foreign genes into crop plants [12]. So far, insecticidal genes have been identified from wide range of *Bacillus thuringiensis* [13] and many of them especially *cry* genes have been engineered into plants [14]. Compared to other methods such as CRISPR/Cas or TALEN [15], Agrobacterium-mediated transformation is the core tool for manipulation of plant genomes [16,17]. Several pea transformation protocols have been developed using different types of initial explants like epicotyl segments [18], protoplasts [19], lateral cotyledonary meristems [20] and segments of the embryonic axis [21-23] or cotyledonary node segments [24]. However, the rate of pea transformation is rather low [22,23] and there is not still a routine method for the regeneration of transgenic pea plants [21,25]. Therefore, an efficient transformation protocol is needed [22]. The improvement of the regeneration system and the selection of appropriate *Agrobacterium tumefaciens* strain are essential factors to increase the transformation efficiency [26].

In this report, the factors affecting transformation efficiency such as infection time, co-cultivation period and regeneration system were investigated. Regeneration system was optimized by using zeatin in the selection medium.

Materials and Methods

Plant material

Pea seeds (cultivar *Sponsor*) were used after surface sterilization with 70% (v/v) ethanol (EtOH) for 1 min followed by 6% (v/v) sodium hypochlorite (NaOCl) for 10 min. Then the seeds were rinsed with sterile distilled water for 5 times and imbibed overnight at room temperature. Prior to transformation, testa and one cotyledon were removed. Radical tips were cut off and the remaining embryonic axes were sliced longitudinally into two segments. It was avoided making more than two segments, because thin slices often do not survive during co-cultivation period [27].

Culture condition and media

All media consisted of MS salts [28] and B5 vitamins [29]. B5hT co-cultivation medium (supplemented with 5 µM TDZ and 1 µM kinetin) and MST medium (supplemented with 5 µM TDZ (Thidiazuron) and 0.01 µM NAA (1-Naphthaleneacetic acid)) were used for T-DNA delivery and enhancing multiple shoot induction respectively. The shoots were selected on P2 medium (supplemented with 20 µM BAP (6-Benzylaminopurine), 0.1 µM NAA and 4.5 µM Zeatin) and increasing concentrations of PPT (2.5, 5, 7.5, 10 mg/l) as a selection agent. Bacterial growth was inhibited by adding 100 mg/l ticarcillin in the selection medium. The pH was adjusted to 5.8 and sterilized by autoclaving at 121°C for 20 min. Explants were maintained in a growth chamber under 16L/8D conditions at 22 ± 2°C. For all media components and recipe see Richter et al. [30].

Agrobacterium strain and bacterial vector

Agrobacterium tumefaciens strain EHA105 was used to generate putative transgenic pea plants using pGII35S-Cry1Ac vector (Figure 1).

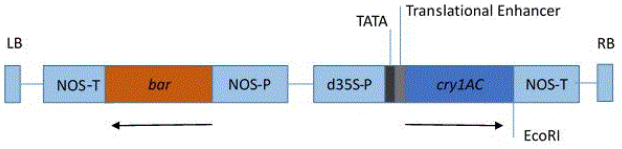

Figure 1: Physical map of binary vector used for pea transformation.

The vector harbored codon optimized insect resistant *cry1Ac* gene from *Bacillus thuringiensis* [31,32] and herbicide resistant *bar* gene from *Streptomyces hygroscopicus* [33]. These transgenic plants were developed at the Department of Plant Biotechnology (Institute of Plant Genetics, Leibniz University of Hannover) with the transformation protocol according to Schroeder et al. [21] and Richter et al. [30] with some modifications.

Transformation procedure

Agrobacterium tumefaciens strain EHA105 was grown in 25 ml yeast extract peptone (YEP) medium (10% Peptone, 10% Yeast extract, 5% Sodium chloride, pH 7.0) supplemented with 50 mg/l kanamycin and 250 µl bacterial stock in a 100 ml Erlenmeyer flask. The flask was placed on a rotatory shaker (200 rpm) at 28°C for 15 h. The bacteria were harvested by centrifugation (4000 rpm, 10 min) at 4°C. The pellets were re-suspended with B5-I medium (Gamborg B5 medium, 10 g/l glucose, 10 g/l sucrose, 2 g/l MES, pH 5.6) to adjust the density (OD_{600} 0.9-1.0). Prepared pea embryos segments were inoculated with Agrobacterium suspension containing 100 µM acetosyringone (a flavonoid that induce the Agrobacterium *vir*-Genes and enhances the transformation efficiency) and 5 µM TDZ for 60, 70 and 90 min in independent experiments. Embryonic segments were then blotted on sterile filter papers and placed on B5hT co-cultivation medium in the dark for 1, 2, 3 and 4 days. After completion of co-cultivation period, explants were washed 5 times with sterile distilled water and the final wash was supplemented with 100 mg/l ticarcillin to eliminate Agrobacterium by incubating for 15 min on a shaker. Explants were dried on sterile filter papers and after decapitation at the base were cultured on MST medium (supplemented with 5 µM TDZ and 0.01 µM NAA) for 10 days (3 days under dim light and 7 days in light). MST medium lacks selection agent to improve the regeneration of infected explants and avoid tissue necrosis [34]. The emerging fresh shoots were sub-cultured every 2 weeks on selective P2 medium (supplemented with 20 µM BAP and 0.1 µM NAA) and gradual increasing concentrations of PPT to eliminate non-transformed cells [35]. Secondary bacterial infection was suppressed by washing the shoots in 100 mg/l ticarcillin and 200 mg/l cefatoxim. In severe infections, the selection medium was supplemented with higher concentration of ticarcilin.

Putative transgenic shoots were micro-grafted to set seeds. Therefore, pea seeds were sown in pots and initially exposed to light for 2 days. Then, the pots (to use as rootstocks) were grown for 6-10 days in the dark. Rootstocks were excised at the tip and a "V" shaped cut was made at the end of scion. Instantly, scions were inserted into the slit of rootstocks and the graft union was wrapped with a tape to fasten the graft edges. Plants were covered with plastic bags to avoid desiccation and placed in a growth room at 22 ± 2°C. Plants were acclimatized within 30 days and thereafter the plastic bags were removed.

Transformation was done with a total of 2232 explants with 7 replicates. All shoots regenerated from a single embryonic segment were considered as clonal plants. Transformation efficiency was calculated as follows:

$$Transformation\ \ efficiency = \frac{Number\ \ of\ \ confirmed\ \ positive\ \ clones\ \ by\ \ PCR}{Total\ \ number\ \ of\ \ initial\ \ clones\ \ \ on\ \ 2.5\ \ mg/ml\ \ PPT} * 100$$

Detection of transgenic plants by PCR

Genomic DNA was extracted using a NucleoSpin® Plant II based on the CTAB and SDS methods. PCR was performed to detect the cry1Ac gene in the transgenic pea plants. The primer sets used for PCR are shown in Table 1.

Primers of HMG-I/Y gene (high mobility group protein) were used to confirm presence of DNA [36]. Chimeric explants were excluded using *A. tumefaciens* specific gene (Pic A-gene).

PCR was done using the following conditions: initial denaturation at 94°C for 5 min, then 29 cycles of denaturation at 94°C for 1 min, annealing at 58-60°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplified PCR fragments were separated on a 1% agarose gel and observed under UV light. Table 1 show the primers used in PCR for the specific genes.

Primers	Sequence	Product Size
Cry1A(c)F	5'-GTTCAGGAGAGAATTGACCC-3'	750 bp
Cry1A(c)R	5'-CTTCACTGCAGGGATTTGAG-3'	
BarF	5'-CTACCATGAGCCCAGAACGACG-3'	447 bp
BarR	5'-CTGCCAGAAACCCACGTCATGCCAGTTC-3'	
HMG-I/Y F	5'-ATGGCAACAAGAGAGGTTAA-3'	570 bp
HMG-I/Y R	5'-TGGTGCATTAGGATCCTTAG-3'	

Table 1: Primers used for amplification of *Cry1A(c)*, *Bar* and *HMG-I/Y*.

Results and Discussion

Effect of inoculation time

Embryo segments were incubated with Agrobacterium suspension for 60, 70 and 90 min. Prolonged immersion resulted in higher transformation efficiency. The transformation efficiency was low (0.99%) after inoculation treatment for 60 min, whereas long inoculum treatment for 90 min increased the transformation efficiency significantly (3.88%-7.89%) (Table 2).

Exp	No. of explants inoculated with Agrobacterium suspension	Inoculation time (min)	Co-cultivation period (days)	Transformation efficiency (%)
A	251	70	1	2.78
B	76	90	2	7.89
C	206	90	3	4.3
D	632	90	4	3.95
E	453	90	4	4.63
F	202	60	4	0.99
G	412	90	4	3.88

Table 2: The influence of inoculation time and co-cultivation period on pea transformation efficiency in different experiments.

Our protocol showed higher transformation efficiency compared to short inoculum period for 30-60 min which gave only 1.5-2.5% [21] and 1% [18]. The infection time plays a key role in Agrobacterium-mediated transformation. While short-term infection is not advantageous for bacterial invasion, a treatment for too long time results in necrosis and consequently a diminution in T-DNA transfer [37].

Effect of co-cultivation time

Co-cultivation time along with infection time influenced the transformation efficiency (Table 2). While co-cultivation for 1 day increased transformation efficiency to 2.78%, the rate plummeted to 0.99% after 4 days treatment at a relatively same inoculation period (60-70 min). In contrast, co-cultivation for 2 days increased transformation efficiency remarkably to 7.89% after a long-term inoculum treatment (90 min). There was no major difference between results where the segments co-cultivated for 3 or 4 days at the same infection time (90 min). Similarly, co-cultivation for 2 days found to be optimal for establishing a system for pea transformation [24] and also for other legumes such as *Vigna radiata* [38,39], *Cajanus cajan* [40] and *Glycine max* [41]. Contrarily, a co-cultivation period of 4 days was more suitable in grass pea (*Lathyrus sativus* L.) [42].

Co-cultivation is an essential step in the transformation process, because bacterial attachment, transferring and integration of T-DNA take place during this stage. The variations in co-cultivation period are resulted due to tissue types, Agrobacterium strains and also the medium used for bacterial culture and co-cultivation [42].

Influence of selective factor

There was a gradual decline in the shoot regeneration frequency after increasing PPT concentration. The regeneration frequency was altered retrogressively and was perceptible on 5 mg/l PPT. The lowest shoot formation and shoot growth was discerned in selection medium supplemented with 10 mg/l PPT. Therefore, the regenerated shoots on 10 mg/l PPT were considered as putatively transformed plants (T_0) and micrografted (Figure 2). No morphological differences were observed between transgenic and non-transgenic plants during the selection, as it was reported previously [23,26].

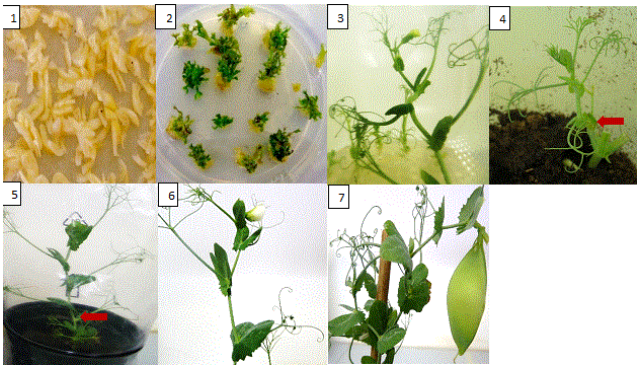

Figure 2: Procedure of transformation. 1: Sliced embryos (inoculated with Agrobacterium). 2: Shoots formed on 2.5 mg/l PPT. 3: Putative transgenic shoots on 10 mg/l PPT. 4: Grafted shoot (arrow shows connection of scion and root stock). 5: Growing of the grafted shoot covered with a plastic bag. 6 and 7: Flower and seed of transgenic plant (T_0).

Transformation efficiency in this study varied from 0.99 to 7.89% compared to 0.7-4.1% in pea (*Pisum sativum*) [26] and 1.29-3.33% in chickpea [43] which phosphinotricin was used in selection medium. Phosphinotricin has been used for the selection of transformed legume crops such as pea [20], soybean [44] and black gram [45].

In contrast, higher transformation rates in pea were achieved with antibiotic selection pressure like kanamycin and hygromycin. Kanamycin resulted in rates from 3.4% [25] to 5% [18] or 8.2% [46]. The rates varied from 4.9% [18] to 15% [19] when hygromycin was used. Compared to antibiotics as selection agents, phosphinotricin has an advantage over kanamycin, because it decreases the regeneration of chimeric plants. Furthermore, the recovery of transgenic plants is increased and the number of escapes is minimized [47]. Therefore, the

herbicide-resistant transgenic plants are more suitable for commercial use [26,46].

Effect of zeatin on regeneration of transgenic plants

Zeatin in combination with BAP and NAA influenced significantly the regeneration frequency. It accelerated shoot induction as well as shoot elongation after 3 weeks. Adding zeatin in selection medium led to 5-6 folds more lateral shoots in more than 80% of total explants. The importance of BAP in legumes for shoot bud differentiation has been reported previously [48]. Addition of NAA (2.7 µM) onto medium containing BAP (22.2 µM) increased frequency of shoot bud regeneration [49]. In the present study, zeatin remarkably improved the effect of BAP and NAA on lateral shoots, shoot induction and its elongation. Higher frequency of shoot induction from embryo axis was observed on pigeon pea using zeatin (1.4 µM) and kinetin (0.93 µM) [50] compared to 2.27 µM TDZ [51]. In strawberry, shoots elongated well [52] and their proliferation was increased on a medium with 1 or 2 µM zeatin [53]. Similarly, zeatin was very effective for shoot induction and shoot elongation of lingonberry (*Vaccinium vitis idaea*) [54].

In contrast, high percentages of shoot production (87.5%) were achieved from embryo derived callus cultures of cowpea (*Vigna unguiculata* L.) on 4 mM BAP [55]. Supplementing both co-cultivation and shoot regeneration media with 10 µM BAP raised transformation efficiency in bean explants [44,56] and in *Pisum sativum* [21] substantially. In cucumber, although using zeatin resulted in higher number of shoot/explant and higher average shoot length compared to BAP, but more than two-fold increase in number of shoot/explant was observed when kinetin was used [57].

In our study, an average number of shoots/explants on P2 selection medium supplemented with zeatin increased (6 fold) compared to the medium without zeatin. About 20% of explants could even generate 35-40 shoots/explant on medium containing zeatin. Similarly, in *Solanum americanum*, the mean of shoots per explant was higher on zeatin/NAA compared to BAP/NAA at the same concentrations [58]. Higher transformation efficiency in tomato was achieved when 4.5 µM zeatin was used alone when compared with the use of BAP alone [59]. Furthermore, zeatin enormously increased the number of shoots/explant in *Andrographis paniculata* and its effect was superior compared to all other cytokinins [60]. Contrarily, higher regeneration efficiency (10 shoots/explant) was reported in pea using embryonic axes slices on the medium containing 10 µM BAP and 10 µM IBA (Indole-3-butyric acid) [61]. In chickpea (*Cicer arietinum* L.), 22 shoots/explant were produced from embryonic axis with half portion of cotyledons at 1.0 µM TDZ concentration [62].

Shoots emerging from selection medium supplemented with zeatin were harder, bigger and in large numbers as it was reported previously [58]. Additionally, the explants exhibited high vigour which resulted in a successful grafting. The grafted shoots were elongated and recovered after one month. Zeatin increased leaf size by 30% of total explants. Explants grown on medium containing zeatin mostly produced flowers after 6-7 cm elongation. Grafting of these budded explants did not produce pod directly and the flowers wilted after 1-2 days. Zeatin accelerated shoot growth and the entire transformation took 4 months, whereas for the explants which zeatin was used from the end of the selection period (10 mg/l PPT in selection medium), it took 7 months to get relatively well-developed shoots for grafting.

In most legumes the efficiency of the shoot multiplication system for genetic modification is low [63]. Transformation efficiency could be improved if the proportion of competent cells in the explants or the number of regenerated shoots from these meristems is increased [64]. To enhance transformation efficiency, zeatin (4.561 µM) as a substituted adenine compound was used in the selection medium to promote shoot regeneration and elongation.

The role of cytokinins and auxins in P2 medium

Cytokinins (like zeatin and BAP) play a prominent role in promoting plant growth and development in the root and shoot meristems [65], cell division and expansion and photomorphogenic development [66]. They also stimulate the initiation and activity of axillary meristems (shoot branching), which results in shoot induction and formation [67]. Although there is a vast knowledge on chemistry of cytokinins, little is known about their mode of action [68]. Auxins (like NAA, a synthetically produced) are responsible for promoting the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin.

The balance of growth regulators depends on the aim of the *in vitro* cultivation such as initiation and multiplication of shoots or roots. In the multiplication phase, the level of citokinins should be normally higher than auxins [69]. Providing good combinations of cytokinins is also crucial to achieve desirable results. In a study on a leguminous plant (*Bauhinia vahlii*), the combination of TDZ and kinetin resulted in a significant increase in shoot numbers [70]. It was shown that the joint use of zeatin and BAP improved shoot elongation and it was more effective than zeatin alone. However, the combined use of zeatin and BAP has also been considered to have the synergistic effect on the morphogenesis of plants [71]. Zeatin is also able to induce chloroplast differentiation and deposits higher total chlorophyll contents. The effect of cytokinins on micro-propagation can be influenced by the kind of culture medium, the variety of plant and the age of explants [72]. On the other hand, the response of explants to the cytokinins depends mostly on the plant genotype [73].

In our study, our data are in agreement with aforementioned results of previous studies. Combination of zeatin and BAP had synergistic effect and gave better results. The number of shoots per explants, shoot elongation, leaves sizes and shoot vigour were apparently affected.

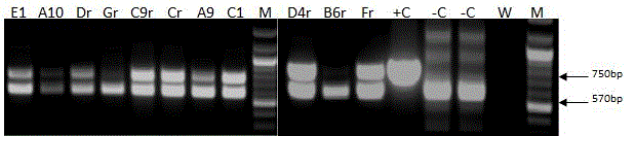

Figure 3: Multiplex PCR amplification of putative transgenic plants (T_0) using primers for *cry 1Ac* gene (product size of 750 bp) and HMG (product size of 570 bp). +C: positive control; -C: negative control; W: water control; M: 100 bp DNA ladder.

Grafting and molecular analysis

The well-developed shoots were successfully grafted on rootstocks in soil and all the grafted plants produced seeds. The major obstacles in regeneration of *in vitro* legumes are root induction and establishing of plantlets in pot or field. Therefore, grafting as an alternative tool was investigated [74,75]. Plants resistant to the phosphinotricin were

analyzed using PCR (Figure 3) where the first screening provided results for further molecular assessments. Presence of *Cry* and *bar* genes in putative transgenic plants and also the results of Southern blot and feeding test for these plants have been reported in our previous publication [76].

Conclusion

In the present study, the Agrobacterium-mediated transformation efficiency was increased by prolonging infection time to 90 min, co-cultivation period for 2 days and improving regeneration efficiency by using zeatin in selection medium. Zeatin improved shoot growth and quality considerably. According to our knowledge, zeatin has not been used in pea transformation yet. This protocol has desirable advantages such as higher transformation efficiency, higher regeneration frequency and shorter duration protocol from seed to seed.

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