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Insect resistant transgenic pea expressing *cry1Ac* gene product from *Bacillus thuringiensis* ☆

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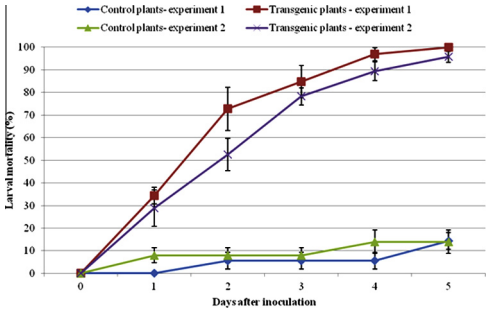
HIGHLIGHTS

- *Agrobacterium*-mediated transformation of pea has been developed.
- T4 generation of transgenic pea plants was confirmed at molecular level.
- Insect resistant transgenic pea expressing cry1Ac protein has been confirmed.
- Total larval mortality was observed on the transgenic plants compared with control.

GRAPHICAL ABSTRACT



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ABSTRACT

Insect pests are the major constraints of grain legumes production and storage. Improvement through conventional breeding strategies has been limited by the lack of resistance traits within the gene pool for most of the economically important pests. This gap can be closed by transgenic approaches using resistance genes from different sources. In this study, we report the development of insect resistant transgenic peas expressing a plant codon optimized *cry1Ac* gene from *Bacillus thuringiensis*. The transgenic nature of regenerated *in vitro* plants and their segregating progenies has been confirmed through molecular analyses (PCR, Southern blot, RT-PCR and immunostrip assay). The introduced transgene was inherited up to the T4 generation. Insect bioassay using larvae of tobacco budworm indicated total larval mortality and significantly reduced feeding damage on the developed transgenic pea plants as compared to 85% larval survival and heavy feeding damage on non-transgenic control plants. The developed transgenic lines can be used for further studies such as gene stacking and field trials.

1. Introduction

Pea (*Pisum sativum* L.), an economically very important multipurpose grain legume, is primarily grown for food and feed throughout the world (Oelke et al., 1991). It is one of the main sources of dietary protein for millions of households (Oelke et al., 1991; Graham and Vance, 2003). Like other legumes, it has the ability to fix atmospheric nitrogen through symbiotic relationship with specific soil bacteria, which makes pea production an important component of the cropping system in order to manage soil fertility (Ferguson et al., 2010).

The production and storage of pea and other grain legumes is constrained by diverse groups of insect pests. Some of the pests affect the growing plants in the field and then contribute to reduced productivity (e.g., pea aphid *Acyrthosiphon pisum*, pea moth *Cydia nigricana*, pea leaf weevil *Sitona lineatus*, etc.) while other insect pests affect the grain during storage and reduce the finally

Abbreviations: bar, bialaphos resistance gene; CTAB, cetyltrimethyl ammonium bromide; PPT, phosphinothricin.

☆ *Key message:* We report the expression of *cry1Ac* gene from *Bacillus thuringiensis* in pea to enhance resistance to field pests. Total larval mortality was observed on the transgenic plants.

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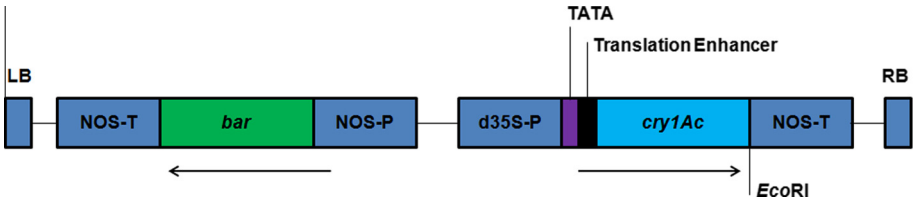

Fig. 1. The physical map of the transformation vector pGII35S-Cry1Ac. d35S-P, double 35S promoter from CaMV; cry1Ac, codon optimized insect resistant gene from *Bacillus thuringiensis* (Sardana et al., 1996; Cheng et al., 1998); NOS-P and NOS-T, *Agrobacterium* nopaline synthase promoter and terminator, respectively; bar, herbicide resistant plant selectable marker gene from *Streptomyces hygroscopicus* (Murakami et al., 1986); RB, right border; LB, left border. Arrows indicate the direction of transcription for the respective genes.

available product for consumption (e.g., most of the weevil species) (Schroeder et al., 1995; Keneni et al., 2011). There are also some insect pests (e.g., pea weevil) which cause yield losses both in the field and during storage (Clement et al., 2002). Significant yield losses by insect pests have been documented by different authors: up to 13% for pea moth, 20–30% for pea aphids, up to 40–70% for pea weevil and up to 10% for pea leaf weevil (Schroeder et al., 1995; Clement et al., 2002, 2009; Legowski and Gould, 1960; Biddle and Cattlin, 2001; Williams et al., 1995).

Insect resistance is a trait lacking for most of the economically important insect pests in pea and other grain legumes (Keneni et al., 2011; Clement et al., 2002). This makes improvement efforts very difficult through conventional breeding. This limitation can be overcome by transgenic approaches, i.e., by introducing transgenes for insect resistance from other sources. Preferably, *cry* genes from the soil bacterium, *Bacillus thuringiensis,* are the most commonly used genes for the development of insect resistant transgenic crops (Korth, 2008). There are different groups of *cry* genes that are active against specific groups of insect pests: *cry*1 group against *Lepidopteran* pests and *cry*3 group against *Coleopteran* pests (Hofte and Whiteley, 1989; Crickmore et al., 1998). Efforts on the development of insect resistant transgenic crops have been reviewed by many authors (Zaidi et al., 2012; Schuler et al., 1998; Gatehouse, 2008).

Some of the specific *cry* genes used so far include the *cry1Ab* gene against maize corn borer in maize (Carozzi and Koziel, 1997), *cry1Ab/cry1Ac* genes against cotton bollworm in cotton (Perlak et al., 1990) and against stem borers in rice (Cheng et al., 1998), and *cry3A* gene against potato beetle in potato (Perlak et al., 1993).

There are numerous reports on transgenic pea development mainly against diseases (Hassan et al., 2009; Richter et al., 2006). The only report to our knowledge on insect resistance was the transgenic pea expressing a bean alpha-amylase inhibitor and the transgenic seeds exhibited resistance against the principal insect pest, pea weevil (Schroeder et al., 1995). However, this has not reached consumers due to observations of an immune response to the expressed alpha-amylase inhibitor in mice tests (Prescott et al., 2005), although a recent report revealed that this apparently is not the case (Lee et al., 2013). In general, however, little attention has been given to the development of insect resistance in pea. So far, there is no report on transgenic pea expressing *cry* genes to improve insect resistance.

The different groups of cry toxins provide a practical and immediate solution to the problem. The major field pests in the order *Lepidoptera* can be addressed by developing transgenic pea expressing a cry1 toxin while the cry3 toxin can be used to target the major storage pests in the order *Coleoptera*. Furthermore, these cry toxins can be stacked into single pea plants so that both the field and storage pests can be controlled.

In this study, we report the development of insect resistant transgenic pea expressing a synthetic plant codon optimized *cry1Ac* gene. The genomic integration, inheritance and expression of the introduced *cry1Ac* gene has been confirmed through molecular analysis while the insect bioassay showed the resistance of the developed transgenic pea lines against one of the target insects.

2. Materials and methods

2.1. Plant materials and transformation vector

In vitro putative transgenic pea *(P. sativum* L. cv. Sponsor) plants developed through *Agrobacterium*-mediated transformation with a transformation vector pGII35S-Cry1Ac (Fig. 1) harboring codon optimized insect resistant *cry1Ac* gene from *B. thuringiensis* (Sardana et al., 1996; Cheng et al., 1998) and herbicide resistant *bar* gene from *Streptomyces hygroscopicus* (Murakami et al., 1986) were used. These putative transgenic plants were developed at the Department of Plant Biotechnology (Institute of Plant Genetics, Leibniz University of Hannover) based on the transformation protocol developed by Schroeder et al. (1993) with modification after Richter et al. (2006). Fig. 2 shows an overview of transgenic pea development steps. Seeds were surface sterilized in 70% ethanol for one minute followed by 6% sodium hypochlorite for 10 min. Then, the seeds were washed 4–5 times with sterilized distilled water and imbibed overnight. The next day, embryos were sliced longitudinally and inoculated with *Agrobacterium* suspension after adjusting the OD_{600} to 1–1.2 for 60 min. After 3–4 days of co-cultivation, the explants were washed thoroughly in distilled water and then in antibiotic solution to eliminate the *Agrobacterium* growth. Subsequently, the explants were transferred to shoot induction medium for 10 days and finally transferred to selective regeneration medium where the regenerated shoots were subjected to increased concentration of selection agent (PPT: 2.5, 5, 7.5 and 10 mg/L) every three weeks. In order to recover the putative transgenic shoots for further molecular and functional analyses, the *in vitro* putative transgenic shoots were micro-grafted (Pickardt et al., 1995) onto seedling rootstock raised on soil substrate until flowering and setting the T0 seeds. Leaf samples were collected from successfully grafted and well grown plants for molecular analysis.

2.2. DNA isolation and PCR analysis

Genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1990). The isolated DNA was used for PCR and Southern blot analyses of the putative transgenic plants and their subsequent progenies.

The PCR program contained the initial denaturation step of 94 °C, 5 min followed by 30 cycles of [94 °C, 1 min denaturation step; 1 min annealing step (Table 1); and 72 °C, 1 min extension step] and the final extension steps of 72 °C, 10 min. Primers for *hmg-I/Y* gene (high mobility group protein) were used as internal control to check the presence of DNA (Gupta et al., 1997).

2.3. Southern blot analysis

Total DNA for Southern blot analysis was isolated from young leaves of transgenic plants using CTAB method (Doyle and Doyle, 1990). The DNA (20 µg) was digested with *Eco*RI, and the resulting fragments were fractionated by electrophoresis on a 0.8% agarose