Transgenic Insect Resistance in Grain Legumes

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

M.Sc. Alemayehu Teressa Negawo

geboren am 31.12.1981 in Showa, Ethiopia

2015

Referent: Prof. Dr. Hans-Jörg Jacobsen

Korreferent: Prof. Dr. Edgar Maiss

Tag der Promotion: 19. 03. 2015

Abstract

Grain legumes or pulses are cultivated throughout the word and play crucial roles in nurturing millions. Moreover, grain legumes can fix nitrogen in symbiosis with *Rhizobia* and thus contribute to maintaining soil fertility in agricultural production systems. However, production and storage of pulses is negatively affected by many factors including insect pests. Grain legumes are attacked by diverse groups of insect pests both in field and during storage after harvesting. Resistance breeding is one of the strategies to reduce yield losses due to insect pests. In grain legumes, however, the lack of resistance genes has limited the successful application of conventional breeding. Transgenic approaches can therefore provide the best alternative in grain legume resistance breeding.

Hence, this study was done with the main goal of improving insect resistance in grain legumes via *Agrobacterium*-mediated transformation. The study was conducted on two important grain legume species (*Pisum sativum* L. and *Vigna unguiculata* L.) with their respective specific research activities and objectives.

In the first part of this study, transgenic pea lines expressing the *cry1Ac* gene from *Bacillus thuringiensis* were grown under growth chamber and greenhouse conditions and used for molecular and functional characterizations. The result demonstrated the stable integration, inheritance and expression of the *cry1Ac* transgene. The inheritance of the transgene was confirmed in advanced generations (T4-T7) of different transgenic lines. Quantitative real-time PCR analysis showed variation in the expression folds (up to 4.72) of *cry1Ac* gene among the different transgenic lines. In the insect bioassay studies, high levels of larval mortality (up to 100 %) and substantially reduced feeding damage were recorded on the transgenic plants from different transgenic lines. The transgenic lines could play a vital role in pea production and improvement programs. However, further studies are required to evaluate the performance of the transgenic lines under field conditions and select the promising ones for future use.

In the second part of this study, regeneration and transformation of cowpea has been pursued. Since there is no routinely and universally applicable protocol for regeneration and *Agrobacterium*-mediated transformation of cowpea, different *in vitro* conditions were optimized using the Kenyan cowpea variety K80. MSB_5 medium supplemented with 3 µM

BA and 0.5 µM Kin was optimal for multiple shoot regeneration from CN explants. A better transformation efficiency (56 % or more) was obtained with embryo explants and inoculation/co-cultivation medium containing 100 µm acetosyringone. The supplementation of the inoculation/co-cultivation medium with 1 mM Na-thiosulphate and a high concentration of acetosyringone (200 µM) improved the transformation efficiency by nearly 40 %. PCR analysis of the putative transgenic shoots showed presence of transgene (*cry1Ac* and *bar* genes) fragments in the genomic DNA of two *in vitro* shoots indicating the genomic integration of the T-DNA region. On the other hand, stable transformation was not achieved in the rest of the experiments. Given the recalcitrance of the crop, further exploration of the *in vitro* conditions and alternative protocols are required to develop a robust protocol that works across genotypes.

Key words: Legumes, insect pests, insect resistance, recalcitrance, regeneration, transformation, Agrobacterium, Bacillus thuringiensis, transgenic approaches

Zusammenfassung

Körnerleguminosen warden welt weit angebaut und konsumiert. Darüberhinaus lesiten Lguminosen durch ihre Fähigkeit zur symbiontischen N_2-Fixierung einen enormen Beitrag zur Verbesserung und Erhaltung der Bodenfruchtbarkeit. Allerdings sind Anbau und Lagerung von Körnerleguminosen durch eine Reihe gefährdet, darunter auch Insektenfraß durch eine Reihe unterschiedlicher Insektengattungen. Da in den Genpools der moisten Leguminosenarten Resistenzgene gegen Insekten fehlen, stößt die klassische Resistenzzüchtung auf unüberwindliche Hindernisse, so daß gentechnische Methoden eingesetzt warden müssen.

In der vorleigenden Arbeit wird versucht, mittels Agrobacterien-vermittelte Transformation bei zwei wichtigen Leguminosenarten (*Pisum sativum* L. und *Vigna unguiculata* L.) die Resistenz gegen Insekten zu verbessern.

Im ersten Teil der Arbeit werden Erstellung und molekulare sowie funktionale Analyse transgener Erbsen vorgestellt, die ein *cry1Ac* Gen aus *Bacillus thuringiensis* exprimieren. Es kann gezeigt warden, daß die Transgene stabil integriert im Genom vorliegen und auch stabil vererbt (bis zur T7) und exprimiert warden. Mittels qPCR kann auch demonstriert warden, dss die unterschieldichen events, die den Linien zu Grunde liegen, zu unterschiedlichen Expressionsniveaus führen (bis zum 4,72-fachen). In Fütterungsstudien wurden hohe Mortalitätsraten von Insektenlarven sowie reduzierte Level an Fraßschäden gefunden. Inwieweit diese aus Pflanzen, die in Gewächshäusern herangezogen wurden, gewonnenen Daten sich übertragen lassen, muss in Freilandversuchen untersucht warden.

Im zweiten Teil der Arbeit sollte untersucht warden, ob sich die Erfahrungen mit der Erbse auf die in Afrika sehr wichtige “cowpea” übertragen lassen. Da diese Species sehr schwierig für *in vitro*-Arbeiten ist, mussten zunächste die Bedingungen für die Regeneration und die Transformation etabliert werden. Als Modell stand die kenianische Sorte K80 zur Verfügung. Es konnten optimierte Kultur- sowie Transformationsbedingungen etabliert warden. So erwiesen sich die Zugaben von Na-thiosulphate und hohen Konzentrationen an Acetysyringon als förderlich. Es konnten im Verlauf des Projekts transgene Sprosse erzeugt und analysiert werden, erste Daten deuten auch auf eine stabile Integration der Transgene hin.

Key words: Legumes, insect pests, insect resistance, recalcitrance, regeneration, transformation, Agrobacterium, Bacillus thuringiensis, transgenic approaches

This dissertation is dedicated to my parents!

Table of contents

Abbreviations

µg:	Microgram
µl:	Microliter
µM:	Micromolar
2,4-D:	2,4-Dichlorophenoxyacetic acid
αAI:	alpha amylase inhibitor
ABA:	Abscisic acid
B5:	Gamborg medium
B5hT:	Co-cultivation medium (pea transformation)
B5i:	Bacterial resuspension medium (pea transformation)
BAP:	6-Benzylaminopurine
bar:	Bialaphos resistance
bp:	Base pair
B.t*:*	*Bacillus thuringiensis*
CaMV:	Cauliflower mosaic virus
CCM:	Cowpea co-cultivation medium
cDNA:	Complementary DNA
CI:	Chloroform: isoamyl alcohol mix
CN:	Cotyledonary node
CTAB:	Cetyltrimethylammonium bromide
DE:	Decapitated embryo
ddH_2O:	Double distilled water
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxyribonucleotide triphosphates
DTT:	Dithiothreitol
EIQ:	Environmental impact quotient
g:	Gram
GFP:	Green fluorescent protein
GM:	Genetically modified
GUS	beta-glucuronidase
HGM:	High mobility group

IBA:	Indole-3-butyric acid
Kin:	Kinetin
LN:	liquid nitrogen
MES:	2-(N-Morpholino) ethanesulfonic acid
mg:	Milligram
Min:	Minute
ml:	Milliliter
mg/L:	Milligram per liter
mM:	Millimolar
M-MuLV:	Moloney Murine Leukemia Virus
MS:	Murashige and Skoog medium (1962)
MSB_5:	MS nutrient medium with B5 vitamins
NAA:	1-Naphthaleneacetic acid
nos*:*	nopaline synthase
NptI:	Neomycin phosphotransferase I
OCS:	Octopine synthase gene
OD:	Optical density
PCR:	Polymerase chain reaction
PGIP:	Polygalacturonase inhibiting protein
PPT:	Phosphinothricin
qRT-PCR:	Quantitative real-time PCR
RNase:	Ribonuclease
rpm:	Revolution per minute
RT-PCR:	Reverse transcriptase polymerase chain reaction
SDS:	Sodium dodecyl sulfate
SIM:	Shoot induction medium
T0:	Putative transgenic clones
T-DNA:	Transfer DNA region of transformation vector
TDZ:	Thidiazuron
TE (%):	Transformation efficiency (%)
TE buffer:	Tris-EDTA buffer
Vst1:	Stilbene synthase
YEP:	Yeast Extract Peptone

List of Tables

List of Figures

1. General introduction

1.1 General information on grain legumes

Grain legumes or pulses are dicotyledonous plants and belong to the Fabaceae/leguminosae family (Rubatzky and Yamaguchi, 1997). With about 450 genera and over 12 thousand known species (Rubatzky and Yamaguchi, 1997), legumes are the third largest family of higher plants (Gepts et al., 2005). The family includes many economically important food crop species such as peas, cowpea, common bean, soybean and peanut (Rubatzky and Yamaguchi, 1997; Gepts et al., 2005).

Legumes have a number of interesting features: they are annual or perennial plants with alternate leaves (pinnate, trifoliate, or digitate) and perfect flower structure (Rubatzky and Yamaguchi, 1997). They exhibit both hypogeal and epigeal germination. In some legumes such as pea, there is a limited elongation of the hypocotyl resulting in hypogeal germination while in other legumes like cowpea and common bean, the elongation of the hypocotyl pushes the cotyledons up to the surface of the soil resulting in epigeal germination (Rubatzky and Yamaguchi, 1997). Another important feature of legumes is their ability to undergo symbiotic relationship with soil bacteria to fix atmospheric nitrogen (Ferguson et al., 2010).

1.2 Importance of grain legumes

In terms of agricultural importance, cereals are only the crops that outweigh legumes (Graham and Vance, 2003; Jaiwal and Singh, 2003; Gepts et al., 2005). Cultivated throughout the world, legumes are a multiple purpose socio-economically important crops. They are used as food, feed, oil, forage, fuel, wood, fiber, ornamentals, green manure and ground cover (Rubatzky and Yamaguchi, 1997; Jaiwal and Singh, 2003; Somers et al., 2003; Varshney et al., 2009). As food crops, they are an important source of good dietary proteins, processable oil and other nutrients (Gepts et al., 2005) for millions of people in many parts of the world. The protein content of grain legumes ranges from 18 to 48 %

(Ranalli, 2003) making them the cheapest sources of daily protein intake in many developing countries. They are also a crucial component of livestock feed as a protein source in both tropical and temperate farming communities of the world (Young et al., 2003).

By symbiotically interacting with soil bacteria (*Rhizobia spp.)*, legumes are also involved in biological atmospheric nitrogen fixation and convert it into a biologically useful form (Ferguson et al., 2010). The fixed nitrogen can be used by the plant itself and/or enrich the soil fertility for the next cropping season. Moreover, legumes can be used as a cover crop to reduce soil erosion, control weeds and conserve soil moisture (Giller, 2001). By the virtue of these characteristics, the production of legumes plays a key role in the sustainability of agricultural production system (Jaiwal and Singh, 2003; Dita et al., 2006).

Being one of the best studied plant families (Young et al., 2003), legumes have been the subject of various genetic, biochemical and physiological studies for many decades (Bean et al., 1997; Young and Bharti, 2012). Mendel has used pea in his studies and developed the principle of inheritance. Legumes like *Medicago truncatula* or *Lotus corniculatus* are considered as a model plants to study the interaction of plants to its environment ranging from biotic to abiotic factors (Gepts et al., 2005; Li et al., 2012).

1.3 Insect pests of grain legumes and their economic importance

Despite their socio-economic and environmental importance, improvement in the yield of legumes has been lagging behind particularly compared to cereals (Graham and Vance, 2003). The yield and productivity of legumes is severely affected by many factors. Biotic factors include fungi, bacteria, viruses, weeds, insects and nematodes while abiotic factors include drought, freezing, salinity, heat, water logging and mineral toxicities (Nene and Reed, 1994; Graham and Vance, 2003; Dita et al., 2006).

Of the biotic factors, grain legumes are attacked by diverse groups of insect pests (Sharma et al., 2010). Like in other crops, insect pests cause both direct and indirect damage to grain legumes (Dita et al., 2006). Direct damage is caused by feeding on the pod and seeds, defoliating the plants and sucking the sap of the plants (Edwards and Singh, 2006; de Filippis, 2012) which eventually result in yield losses in terms of quantity and quality. Insect

pests also act as vectors for viral infections inflicting indirect damage to crop plants (Christou and Twyman, 2004).

Insect pests in general cause yield losses both in the field as well as during storage after harvesting (Christou and Twyman, 2004). Despite heavy use of chemical insecticides, about 37 % of world crop production is lost to diseases and pests, with at least 13 % lost directly to insect pests (Gatehouse et al., 1993). In grain legumes, up to 100 % yield losses can be caused by insect pests both in the field and/or during storage depending on the legume species, the insect pest type and the location (Clement et al., 2000; Sharma et al., 2010).

In the field, legumes are damaged by insect species of the orders Lepidoptera, Diptera, Homoptera, Heteroptera and Coleoptera (Clement et al., 2000; Sharma, 2008; Sharma et al., 2010). Some of the economically important pests include pod borers, leaf miners, weevils, aphids, whitefly, leafhoppers and thrips (Saini and Sharma, 2013).

During storage, legumes are attacked by seed beetles (Coleoptera: Bruchidae) (Credland, 1994). In the family Bruchidae, about 20 species are known to be storage pests of legumes (Credland, 1994). Some of the economically important species include *Callosobruchus maculatus*, *C. chinensis*, *C. analis*, *C. rhodesianus*, *C. subinnotatus*, *Acanthoscelides obtectus*, *Zabrotes subfasciatus*, *Bruchus pisorum*, *B. rufumanus* and *Bruchidius atrolineatus* (Pajni and Gill, 1991; Credland, 1994).

1.4 Resistance breeding in grain legumes

The desirable way to combat production constraints is to use integrated approaches (Nene and Reed, 1994) that combines two or more compatible control measures. This can include host plant resistance, cultural practices, chemicals and natural enemies (de Filippis, 2012). The use of resistant varieties is an important aspect of integrated approaches. Hence, the development and availability of resistant varieties plays a very crucial role for substantial reduction of yield losses.

1.4.1 Insect resistance breeding in grain legumes

The majority of grain legume species are self pollinated with a low degree of out crossing (Rubatzky and Yamaguchi, 1997; Gepts et al., 2005). Theoretically, breeding strategies (such as backcrossing, pedigree selection, bulk selection, pure line selection and hybrid line development) for self pollinated crops can be used to develop improved varieties (Ambrose, 2008; Koutsika-Sotiriou and Traka-Mavrona, 2008; Keneni et al., 2011). In some legume crops such as cowpea, conventional breeding achieved progress with respect to resistance against bacteria, fungal and viral diseases, parasitic weeds and root-knot nematodes as well as for drought and heat tolerance (Timko et al., 2007; Timko and Singh, 2008; Lucas et al., 2013). In pea, resistance to powdery mildew has been achieved using conventional breeding as well (Fondevilla and Rubiales, 2012).

Development of resistant varieties can however only be achieved by conventional breeding approaches when the trait is available in the gene pool of the species or compatible wild relatives. Unfortunately, for most of the economically important insect pests, resistance traits are lacking in the gene pool of the legume species (Clement et al., 2002; Chaudhury et al., 2007; Keneni et al., 2011). Attempts to transfer from wild relatives (if any) were hampered by crossing incompatibility or co-transfer of unwanted traits (Machuka, 2002; Singh et al., 2002; Popelka et al., 2004; Chaudhury et al., 2007; Keneni et al., 2011).

As state of the art nowadays genetic engineering systems can be applied when resistance traits in the species' gene pool is limited. There are a number of cases where genes for agro-economically important traits (such as insect, diseases and herbicide resistances) where obtained from other sources and transferred to crop plants via transgenic approaches (Korth, 2008). The same approaches can be applied in legume improvement efforts in order to introduced resistance traits against the economically important insect pests.

1.4.2 Transgenic insect resistance

Using genetic engineering techniques, transgenes for traits of interest can be identified, isolated and introduced to the plant genome (Schroeder et al., 2000). For transgenic insect resistance development in crop plants, transgenes have been identified and isolated from

diverse sources ranging from prokaryote species (like bacteria) to eukaryote species (such as fungi and plants) (Christou and Twyman, 2004; Korth, 2008). The protein products from the expressed transgenes have a negative lethal effect on the target insect pests (Korth, 2008), while not harming humans or animals. Candidate transgenes encoding for such proteins such as lectins, protease inhibitors, Chitinase and delta-endotoxins (Jouanin et al., 1998; Korth, 2008) have been identified. Of the different potential transgenes, *cry* genes from the soil bacterium *Bacillus thuringiensis* encoding for endotoxins are the most commonly used transgenes conferring insect resistance (Korth, 2008). Some of the *B.t cry* genes expressed in transgenic plants include *cry1Ac* gene in maize, soybean, chickpea and rice; *cry1Ab* gene in maize, chickpea, tomato and rice; and *cry3a* gene in potato (Perlak et al., 1990; Stewart Jr et al., 1996; Carozzi and Koziel, 1997; Cheng et al., 1998; Mandaokar et al., 2000). Today, insect resistant transgenic crops are the second most popular commercialized traits next to transgenic herbicide resistance (James, 2013).

In insect pest management, control measures are used to disrupt the life cycle of the insect pests. The Cry proteins are targeted to the larval stage of the insect (Bravo et al., 2011). Fig. 1 shows the three dimensional structure of activated endotoxins and the roles played by the different domains on the mode of endotoxins action. To be effective, the delta-endotoxins have to be ingested by the larvae of the target insect. When the larvae feed on the transgenic B.t plant, the ingested Cry proteins are activated in the midgut of the larvae and bind to the receptors on the epithelial cell membrane (domain II and III are suggested to be involved in the action) leading to the formation of membrane pores with the involvement of domain I. The formation of lytic pores causes the uncontrolled release of ions, the collapse of the epithelia that stops the larvae from feeding and finally leads to the death of the larvae (de Maagd et al., 2001; Bravo et al., 2007).

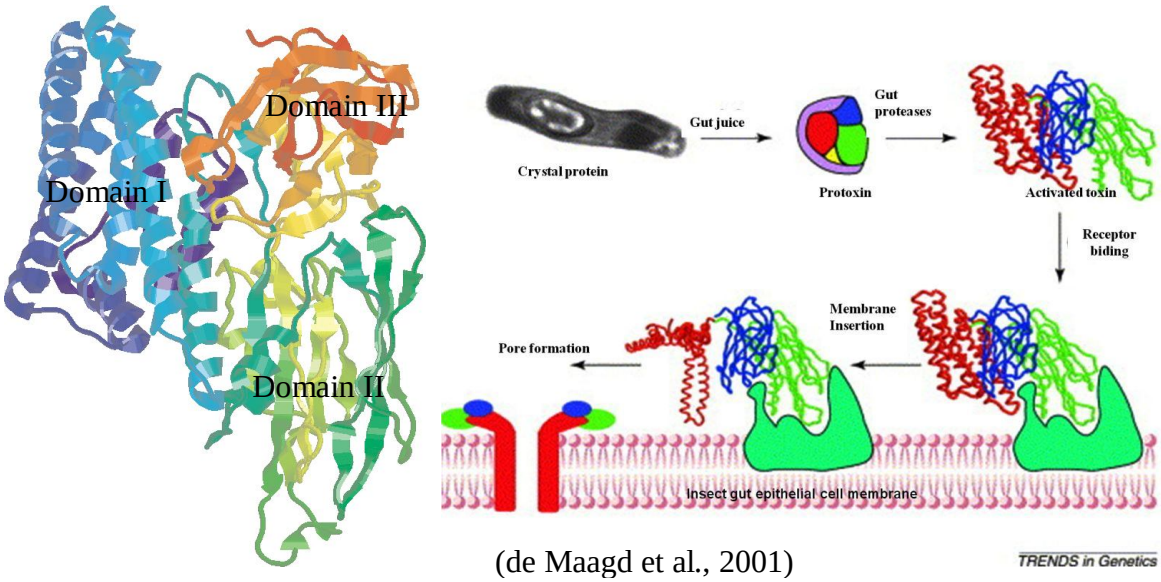


Fig. 1 Three dimensional structure (left) of the activated endotoxins and the mode of action (right) of the endotoxins. During the process of action, domains II and III are involved in binding to receptors on the epithelial cell membrane while domain I is involved in pore formation (de Maagd et al., 2001).

1.5 Regeneration and genetic transformation in grain legumes

Plant breeding has benefitted from the advancement in transgenic approaches. New crop varieties with novel traits have been developed and their production has been realized in many countries throughout the world (James, 2013). These crops include maize, cotton, soybean and canola with novel herbicide tolerance and/or insecticide resistance traits. Despite their socio-economic importance, grain legumes have not yet found the same attention regarding genetic engineering (Popelka et al., 2004; Eapen, 2008). Today, in spite of some reports, there is no commercial production of a single transgenic event in grain legumes.

The success of biotechnological approaches depends on many factors ranging from the plant species itself to the availability of protocols allowing regeneration, transformation and appropriate selection systems (Atkins and Mc Smith, 1997). Like in other crops, efficient regeneration and transformation protocol is a must to apply genetic engineering in grain legumes (Atkins and Mc Smith, 1997). In few grain legumes like pea, there is a well established protocol for regeneration and transformation (Schroeder et al., 1993). However, in some orphan legumes like cowpea, there is no routinely applicable protocol (Brar et al.,

1997b; Chandra and Pental, 2003; Popelka et al., 2006). Legumes belong generally to the group of recalcitrant plants for *in vitro* manipulations (Atkins and Mc Smith, 1997; Brar et al., 1997b; Somers et al., 2003; Chaudhury et al., 2007). In several cases, the existing protocols are variety dependent and require the need to optimize *in vitro* conditions for a given variety in hand (Brar et al., 1997b; Somers et al., 2003). In general, *in vitro* manipulation of grain legumes is a challenging activity (Brar et al., 1997b; Schroeder et al., 2000; Somers et al., 2003; Chaudhury et al., 2007) where further efforts are required in order to develop robust and genotype neutral protocols necessary for the application of genetic engineering.

Therefore, this study was conducted with the general aim of improving insect resistance in two different grain legumes, pea and cowpea, through transgenic approaches using *Agrobacterium*-mediated transformation.

2. General Materials and Methods

2.1 List of equipment

The equipment used in this study is listed in Appendix 1

2.2 List of chemicals

The list of chemicals is indicated in Appendix 2.

2.3 List of buffers and solutions

The buffers and solutions used during the study are listed in Appendix 3.

2.4 YEP media for overnight culture of *Agrobacterium*

10 g/L Tryptone

5 g/L Yeast Extract

5 g/L NaCl, pH 7

2.5 Plant culture media

Murashige and Skoog basal medium (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg et al., 1968) (MSB_5), supplemented with 30 g/L sucrose and 1 g/L MES was used. The medium was adjusted to a pH 5.8 and 7.5 g/l plant agar was added prior to autoclaving for 20 min at 121^{o}C. Heat sensitive components (plant hormones and antibiotics) were filter-sterilized and added to the medium post autoclaving.

2.6 Methods

2.6.1 Overnight culture of *Agrobacterium* and preparation of glycerol stocks

Overnight culture of *Agrobacterium* and preparation of glycerol stocks was done following a standard procedure (Hassan, 2006). A glycerol stock (500 µL) of the *Agrobacterium* with the transformation vector (from -80°C) was cultured overnight in 500 ml YEP medium supplemented with 50 mg/L Kanamycin. The culture was maintained on a shaker (170-200 rpm) at 28°C. The next day, the overnight culture was harvested by centrifugation (4,500 rpm) at 4°C for 10 min. The harvested culture was resuspended in inoculation medium by adjusting the optical density (OD_{600}) to the required level.

Agrobacterium glycerol stock was prepared by mixing 500 µL of 86 % glycerol and 1000 µL of the overnight culture in 2 ml cryogenic vials. The prepared stock was immediately placed in liquid nitrogen and transferred to -80°C for storage.

2.6.2 Plasmid DNA isolation

Plasmid DNA was isolated from an overnight culture of *Agrobacterium* using the alkaline extraction method (Birnboim and Doly, 1979). Two milliliters of overnight culture of *Agrobacterium* were added into a 2 ml microcentrifuge tube and harvested at 12,000 rpm for five min. The supernatant was discarded and the harvesting step was repeated with another 2 ml aliquot of the culture. The harvested pellet was suspended in 200 µl Sol. A (15 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM Glucose, 2 mg/ml fresh Lysozyme) and incubated for 15 min at room temperature. Then, 400 µl Sol. B (0.2 M NaOH, 1 % SDS) and 300 µl Sol. C (3 M NaOAc, pH 4.8) were added and mixed gently, and then incubated for 15 min on ice. After 10 min centrifugation (12,000 rpm), the supernatant was transferred into a new microcentrifuge tube and centrifuged for another 10 min. Then, 800 µl of the supernatant was transferred into a new microcentrifuge tube and 600 µl cold isopropanol was added and mixed gently till the DNA starts to precipitate. After centrifugation for 10 min, the supernatant was discarded and the pellet was dissolved in 200 µl sol. D (0.1 M NaOAc pH 7.0, 0.05 M Tris-HCl pH 8.0), and then 400 µl absolute ethanol were added and mixed. After

10 min centrifugation, the supernatant was discarded and 200 µl 70 % ethanol were added and centrifuged for another 10 min. Then, the ethanol was discarded and the pellet was dried at room temperature. Finally, the plasmid DNA was dissolved in 50 µl TE buffer and stored at 4°C for later use.

The isolated plasmid DNA was checked with PCR using primers for the gene of interest (GOI). Fig. 2 shows a control PCR gel for the isolated plasmid DNA. The isolated plasmid DNA was then used as a positive control during molecular analyses (PCR and RT-PCR) of putative transgenic plants and their progenies.

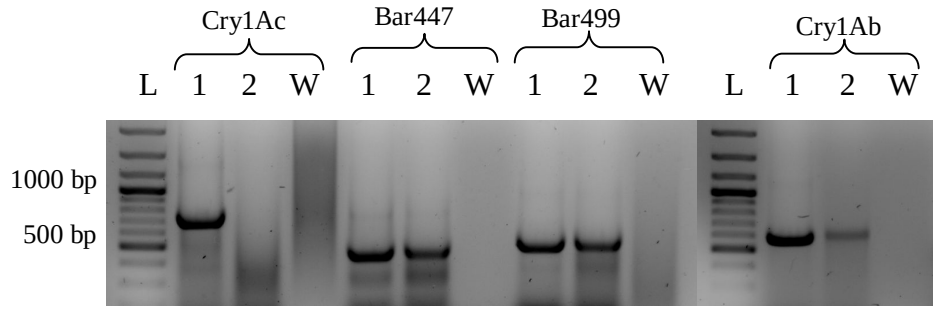


Fig. 2 Control PCR gel for plasmid DNA isolation indicating the specificity of the expected PCR product for the respective plasmid DNA using T-DNA region specific primers. Cry1Ac (750 bp), Bar447 (447 bp) and Bar499 (499 bp) primers were used to amplify gene specific fragment from a plasmid DNA isolated from a transformation vector harboring *cry1Ac* gene while Cry1Ab (600 bp) primers were used to amplify cry1Ab specific sequence from plasmid DNA isolated from a transformation vector harboring *cry1Ab* gene. L: GeneRulerTM 100 bp plus DNA ladder, 1 and 2: Plasmid DNA and W: Water control.

2.6.3 Genomic DNA isolation from leaves

Quick method: A quick and simple DNA isolation protocol (Edwards et al., 1991) was used with little modification as described elsewhere (Kumari et al., 2012). Briefly the steps were as follows:

1. Collect fresh leaves into a 2 ml microcentrifuge tube and grind the tissue using liquid nitrogen (LN) cooled forceps.
2. Add 500 µl extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) and homogenize using vortex at room temperature.

Alternatively, collect fresh leaves in 2 ml micro-tube (Sarstedt, Germany) containing beads and 500 µl extraction buffer, and lyse the leaves using Precellys^{TM} homogenizer. Then directly continue with step 3.

3. Add 500 µL CI-mix and homogenize using vortex at room temperature
4. Centrifuge for 3 min at 10,000 rpm and transfer 500 µl of the supernatant to new 1.5 µl microcentrifuge tube.
5. Add 500 µl isopropanol and mix by inverting the tube and incubate at -20 °C for 10-30 min.
6. Centrifuge for 3 min at 10,000 rpm and discard the supernatant.
7. Wash the pellet with 200 µl 70 % ethanol.
8. Dry the pellet at room temperature (~60 min) or 37°C (~30 min).
9. Dissolve the pellet in 50 µl sterile ddH2O or TE-buffer overnight at +4°C.
10. For PCR amplification, 1-2 µl of the dissolved DNA are used.

CTAB method: For large quantity and good quality DNA isolation, the CTAB-based protocol (Doyle and Doyle, 1990) was used as follows: fresh leaves (8-10) were collected, cooled in LN and grinded with a LN cooled forceps. Then, 5 ml preheated CTAB buffer (60°C) was added to the sample under the fume hood and incubated for 30 min at 60°C in a water bath. Then, 5 ml CI-mix was added and centrifuged for 10 min at 4,500 rpm. After centrifugation, 5 ml of the clear aqueous phase was transferred into new tubes and 3.5 ml isopropanol was added. Then, the mixture was gently mixed and centrifuged for 10 minutes at 4,500 rpm. The supernatant was discarded and the pellet was washed with 2 ml washing buffer. After removing the washing buffer, the pellet was resuspended in 1 ml TE buffer supplemented with RNase (10 mg/ml) and incubated for 30 min at 37°C. Then, 750 µl 7.5 M NH_4-acetate and 2.5 ml absolute ethanol were added successively. After gentle mixing, the mixture was centrifuged at 4,500 rpm for 10 min. Finally, the supernatant was discarded and the pellet was resuspended in 400 µl TE buffer and dissolved overnight at 4°C or stored for later uses. For PCR amplification, 1 µl of the dissolved DNA was used.

2.6.4 Total RNA isolation

From each plant, young leaves (~100 mg) were collected into 2 ml microcentrifuge tubes and placed immediately in LN. Then, the samples were brought to the laboratory and pulverized using LN cooled forceps. Then, total RNA was isolated using NucleoSpin® RNA plant (Macherely-Nagel) according to the manufacturer's instruction.

2.6.5 Complementary DNA (cDNA) synthesis

First strand cDNA was synthesized by RevertAid^{TM} H Minus First Strand cDNA Synthesis kit (MBI Fermentas/Thermo Scientific) according to the manufacturer's instruction. Briefly, a determined amount of total RNA (~5 µg) was mixed with 1 µl Oligo $(dT)_{18}$ primer in a reaction volume of 12 µl with DEPC-treated RNase free water. The mixture was incubated for 5 min at 65°C using a thermocycler and then chilled on ice. Then, 4 µl 5X Reaction buffer, 1 µl RiboLock^{TM} RNase inhibitor and 2 µl dNTP mix were added to the tube, and then mixed and incubated at 37°C for 5 min in a thermocycler. Then, 1 µl RevertAid^{TM} H Minus M-MuLV Reverse Transcriptase was added and incubated at 42°C for 60 min followed by 70°C for 10 min. Finally the reaction was stopped at 4°C and then stored at -20°C for later use. For PCR and qRT-PCR analysis using gene specific primers, 1 µl of the synthesized cDNA was used as template.

2.6.6 Polymerase chain reaction (PCR)

PCR was used to amplify gene specific sequences from the DNA of putative transgenic shoots and their progenies. Table 1 shows the list of primers used during PCR amplification of specific sequence. Table 2 and 3 shows the PCR component preparation and PCR program, respectively.

Table 1. List of primers for PCR analysis

Gene of interest	Primer name	Primer Sequence	Tm (°C)*	Expected Product
cry1Ac gene from *B. thuriengenesis*	Cry1Ac-For Cry1Ac-Rev	5'-GTTCAGGAGAGAATTGACCC-3' 5'-CTTCACTGCAGGGATTTGAG-3'	56	750 bp
cry1Ac gene from *B. thuriengenesis*	Cry160-For Cry160-Rev	5'-GATTGGAAACTACACCGACC-3' 5'-GGAGTCATAGTTCGGGAAGA -3'	59	160 bp
cry1Ab gene from *B. thuriengenesis*	Cry1Ab1025-For Cry1 Ab1635-Rev	5'-CTATGGGAAACGCCGCTCCA-3' 5'-TCCGTCGATGGAGGTGTGGA-3'	60	600 bp
bar gene from *S. Hygroscopicus*	Bar447-For Bar447-Rev	5'-GATTTCGGTGACGGGCAGGA-3' 5'-TGCGGCTCGGTACGGAAGTT-3'	60	447 bp
bar gene from *S. Hygroscopicus*	Bar499-For Bar499-Rev	5'-CTACCATGAGCCCAGAACGACG-3' 5'-CTGCCAGAAACCCACGTCATGCCAGTTC-3'	60	500 bp
A. tumefaciens specific gene	Pic A-For Pic A-Rev	5'-ATGCGGATGAGGCTCGTCTTCGAG-3' 5'-GACGCAACGCATCCTCGATCAGCT-3'	63	550 bp
HMG-I/Y gene for pea**	HMG-For HMG-Rev	5'-ATGGCAACAAGAGAGGTTAA-3' 5'-TGGTGCATTAGGATCCTTAG-3'	56	570 bp/ 370 bp[+]
HMG -I/Y gene for pea	HMGIII-For HMGIII –Rev	5'-AGGGGTAGGCCGAAGAAGAT-3' 5'-TGAGGCTTCACCTTAGGAGG -3'	59	164 bp
HMG gene for cowpea[++]	cHMG-For cHMG-Rev	5′-GCACAGTTTGGGTATATTG-3′ 5′-GTAAAACTGGCAAAAATTAG-3′	56	300 bp
NptI gene	NptI-For NptI-Rev	5'-GAAAAACTCATCGAGCATCA-3' 5'-TTGTCCTTTTAACAGCGATC-3'	53	400 bp
PR10a gene from potato	PR10-For PR10a-Rev	5'-ATGGGTGTCACTAGCTATACACATG-3' 5'-TTAAGCGTAGACAGAAGGATTGGC-3'	57	480 bp
Dreb2a gene from rice	Dreb780-For Dreb780-Rev	5'- AGGGGAGATTGCTCCGTGC-3' 5'- CCCATCATCTCCCTCTTGG-3'	62	780 bp

*Annealing Temperature,**(Gupta et al., 1997),[+]570 bp for genomic DNA and 370 bp for cDNA, [++](Phelps et al., 2007)

Table 2. PCR component preparation

PCR ingredients	DNA isolation method	
	Quick method	CTAB method or cDNA
Double distilled H_2O (autoclaved)	9.3 µl	10.3 µl
10 X PCR buffer (GoTaq-Promega)	5.0 µl	5.0 µl
$MgCl_2$ (25 mM)	2.5 µl	2.5 µl
DMSO	1.0 µl	1.0 µl
Primer1-Forward (10 pmol/ml stock)	1.0 µl	1.0 µl
Primer1-Reverse (10 pmol/ml stock)	1.0 µl	1.0 µl
Primer2-Forward (10 pmol/ml stock)	1.0 µl	1.0 µl
Primer2-Reverse (10 pmol/ml stock)	1.0 µl	1.0 µl
dNTP mix (10 mM)	1.0 µl	1.0 µl
GoTaq DNA polymerase (5U / ml)	0.2 µl	0.2 µl
DNA sample	2.0 µl	1.0 µl
Total volume	**25.0 µl**	**25.0 µl**

Table 3. PCR program

Steps	Temperature	Duration (Min)	No. of Cycles
1. Initial denaturation	94°C	10	1
2. Denaturation	94°C	1	
3. Annealing	Vary*	1	30
4. Synthesis(Extension)	72°C	1	
5. Final Extension	72°C	10	1
6. Pause	4°C	∞	1

*Indicated in Table 1

2.6.7 Gel electrophoresis and documentation

The PCR products were separated on a 1 % agarose gel. The gel was prepared by mixing 1 g agarose in 100 ml 1xTAE buffer and then heating in a microwave oven until the agarose was completely dissolved and the solution looked clear. The heated solution was cooled and Redsafe^{TM} Nucleic Acid staining solution (5 ml for 100 ml) was added and mixed well. The mixed solution was poured into the gel casting box and a comb was inserted at the required distance. After solidification, the gel was placed in the electrophoresis tank filled with 1xTAE buffer sufficient to cover the gel. Then, the comb was carefully removed and the PCR products were loaded into the slots. After all samples were loaded, the lid of the tank was closed and the gel electrophoresis chamber was connected to the power source. The gel was run for 50-60 minutes at 100-120 V. Then, the gel was photographed under UV light and documented.

2.7 Data analysis

Data from the regeneration and rooting experiments were subjected to analysis of variance (ANOVA) and mean separation test using SAS9.2 software.

3. Molecular and functional characterization of Cry1Ac transgenic pea plants and their progenies

Alemayehu Teressa Negawo[1], Linda Baranek[1], Fathi Hassan[1] and Hans-Jörg Jacobsen[1*]

[1]Institute for Plant Genetics (Section of Plant Biotechnology), Gottfried Wilhelm Leibniz Universität Hannover, Herrenhäuser str.2, 30419 Hannover, Germany

[*]*Correspondence address*: Jacobsen@genetik.uni-hannover.de

Section note:

This study was a continuation of my master work. After further molecular and functional characterizations, the following paper has been published on Biological control.

Alemayehu Teressa Negawo[a], Mitra Aftabi[a], Hans-Jörg Jacobsen[a], Illimar Altosaar[b] and Fathi S. Hassan[a*]. 2013. Insect resistant transgenic pea expressing *cry1Ac* gene product from *Bacillus thuringiensis*. Biological Control, 67(3): 293–300. doi: http://dx.doi.org/10.1016/j.biocontrol.2013.09.016

[a]Institute for Plant Genetics (Section of Plant Biotechnology), Gottfried Wilhelm Leibniz Universität Hannover, Herrenhäuserstr.2, 30419 Hannover, Germany

[b]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 452 Smyth Road, Ottawa, ON, Canada.

*corresponding author: fathihassan@hotmail.com

In this section of the thesis, additional data (unpublished, unless otherwise stated) from molecular and functional analyses will be summarized.

3.1 Abstract

Progenies of Cry1Ac transgenic pea lines were characterized at molecular and functional levels. In the PCR, RT-PCR and qRT-PCR analyses, transgene specific primers were designed and used for the amplification of transgene specific fragment. The accumulation of Cry1Ac protein in the tissue of transgenic plants was detected using immunostrip assay specific to Cry1Ab/Cry1Ac proteins. Leaf paint assay using 600 mg/L Basta® herbicide solution was used for functional characterization of the progenies from transgenic lines. Insect bioassay was conducted to evaluate the resistance level of the transgenic lines using tobacco budworm larvae.

The result of molecular and functional analyses showed the presence, inheritance and expression of the introduced transgene at different progeny levels. Variation in the expression levels of the *cry1Ac* gene was observed among the different transgenic lines. The result of immunostrip assay showed the presence of the Cry1Ac protein in plants from the different transgenic lines. In the insect bioassay studies, both larval survival and plant damage were highly affected on the different transgenic plants. Up to 100 % larval mortality was observed on the transgenic plants compared to 17.42 % on control plants. Most of the challenged transgenic plants showed very negligible to substantially reduced feeding damage indicating the insect resistance of the developed transgenic lines. In general, the result confirmed the development of insect resistant Cry1Ac transgenic pea lines which could potentially be used in pea production and improvement programs.

Key words: Pea, cry1Ac protein, insect pests, transgenic resistance, larval mortality, feeding damage

3.2 Introduction

Insect pests are one the main production constraints affecting crop production and productivity throughout the world. Estimated yield losses due to insect pests vary from crop to crop and region to region (Sharma et al., 2010). Pea (*Pisum sativum* L.) is susceptible to many insect pests both in the field and during storage and 10 to 70 % yield losses can be caused depending on the insect pests (Legowski and Gould, 1960; Schroeder et al., 1995; Williams et al., 1995; Biddle and Cattlin, 2001; Clement et al., 2002).

One way to complement conventional breeding approach is to apply modern biotechnology techniques to access genes for novel traits from other sources. This approach has been used to develop not only resistant varieties to production constraints (such as insect and diseases) but also to improve the nutritional value of different crops (Korth, 2008).

Pea is one of the economically important legume crops that have been extensively studied under *in vitro* conditions during the last few decades. Both organogenesis and somatic embryogenesis (Gamborg et al., 1974; Lehminger-Mertens and Jacobsen, 1989; Ozcan et al., 1992) have been used in pea *in vitro* regeneration. Nowadays, organogenesis is the most commonly used regeneration system in pea for *in vitro* manipulations.

Benefited from the decades of *in vitro* studies, pea is one of the few legumes that have been repeatedly genetically modified via transgenic approaches. Of the different plant transformation methods, *Agrobacterium*-mediated is the most commonly used method in pea (de Kathen and Jacobsen, 1990; Puonti-Kaerlas et al., 1990; Schroeder et al., 1993; Bean et al., 1997; Richter et al., 2006; Krejci et al., 2007; Hassan et al., 2009).

Similar to other plants, different types of explants have been explored in pea regeneration such as embryonic axis of immature seed (Schroeder et al., 1993), lateral cotyledonary meristems from germinating seeds (Bean et al., 1997), meristematic tissue initiated from nodal tissue (Tzitzikas et al., 2004), an immature embryo with the embryo axis and the basal part of a cotyledon (Pniewski and Kapusta, 2005) and embryo slices from mature seeds (Richter et al., 2006; Krejci et al., 2007; Hassan et al., 2009). Of these explants,

embryos from mature seeds are probably the cheap and easily available explants in pea regeneration and transformation.

Of the different regeneration and transformation protocols, the protocol reported by Schroeder and his colleagues (Schroeder et al., 1993) is the most commonly used and reproducible protocol for pea regeneration and transformation. In most cases, modification to this protocol was made to the explant sources since immature embryo is not always available. Based on this protocol or its modification, transgenic pea lines have been developed against different production constraints such as insect pests (Shade et al., 1994; Schroeder et al., 1995) and diseases (Richter et al., 2006; Hassan et al., 2009). Some of the transgenes introduced into the pea genome with agronomic importance include *bar*, αAI, *PGIP*, *Vst1* and *Chitinase* genes (Schroeder et al., 1993; Shade et al., 1994; Schroeder et al., 1995; Richter et al., 2006; Hassan et al., 2009). In general, against of the odds of many grain legumes which are recalcitrant to *in vitro* conditions, there is a well established and routinely used regeneration and transformation protocol for pea genetic modification to introduce novel traits.

Despite the successful application of transgenic approach in pea genetic modification, little attention has been given to insect resistance development using B.t *cry* genes. In line with this gap, we have reported the successful development of transgenic pea lines expressing *cry1Ac* gene from *Bacillus thuringiensis* (Negawo et al., 2013). In this study, we characterized and evaluated the insect resistance of the different Cry1Ac transgenic pea lines.

Cry1Ac gene is one of the commonly used B.t *cry* genes to develop transgenic lines in many plant species such as tobacco, cotton, maize, soybean, tomato and rice (Perlak et al., 1990; Stewart Jr et al., 1996; Cheng et al., 1998; Bohorova et al., 1999; Mandaokar et al., 2000). The product of *cry1Ac* gene (Cry1Ac crystal toxin) is active against the Lepidopteran insect pests (Hofte and Whiteley, 1989; Crickmore et al., 1998). The Lepidopteran insect pests are one of the major pests of pea and many legume species which cause significant yield losses under field conditions (Sharma et al., 2010). Their control by conventional measures is inefficient due to different reasons such as absence of resistant variety. Even though chemical pesticides are used at a cost of billion of dollars, an average yield loss of 30 % is still caused by insect pests in legumes (Sharma et al., 2010). Sometimes, it is difficult to

target the insect pests (e.g. larval stage) via pesticide application since they are located inside the crop tissue (Christou and Twyman, 2004). There is also a clear socio-environmental concerns related to the heavy use of chemical pesticides in agriculture production system (Sharma et al., 2000) which needs to be changed. Besides million of chronic illnesses related to pesticide poisoning, about 200,000 people are estimated to be killed per year worldwide by pesticides (Odukkathil and Vasudevan, 2013). Hence, the application of transgenic approaches is a viable alternative strategy to control the insect damage and reduce the load of chemical pesticide used in agricultural system (Christou and Twyman, 2004) and finally increase the crop yield available for consumers.

Thus, the current study has contributed to the development of insect resistant pea plants by analyzing Cry1Ac transgenic pea lines at molecular and functional levels. The results of molecular and functional characterization have confirmed the presence, inheritance, expression and functionality of the introduced *cry1Ac* gene in the developed transgenic pea lines.

3.3 Materials and Methods

3.3.1 Plant material

In this study, seeds of Cry1Ac transgenic pea (*Pisum sativum* L. cv. sponsor) lines (Negawo, 2012) were used for molecular and functional characterizations. They were developed using the routine pea transformation protocol (Schroeder et al., 1993) with modification as described in Richter et al.(2006). Briefly, dry seeds were surface sterilized with 70 % Ethanol for one min followed by 6 % NaOCl solution for 10 min. The seeds were washed 3-5 times with sterile distilled water and soaked overnight in sterile distilled water. The next day, embryo slices (explants) were isolated and soaked in *Agrobacterium* suspension (OD_{600}= 1) for 60 min. Then, the explants were removed from the *Agrobacterium* suspension, blotted dry on sterile filter paper and then cultured on co-cultivation medium for three days in dark. The co-cultured explants were washed (first with water and then Ticarcillin solution), filter paper dried and cultured on multiple shoot induction medium for 10 days under semi-dark condition. Then, the induced shoots were

selectively regenerated on medium supplemented with increased concentrations of PPT (2.5 to 10 mg/L) every 3-4 weeks.

3.3.2 Analysis of transgene stable integration, inheritance and expression

3.3.2.1 PCR and RT-PCR analyses

Cry1Ac transgenic pea lines and their progenies were characterized using PCR as described in materials and methods sections. In PCR confirmed transgenic plants, expression of the transgene at RNA was analyzed using RT-PCR.

3.3.2.2 Quantitative real-time PCR (qRT-PCR)

The expression level of the *cry1Ac* gene was determined using quantitative real-time PCR (qRT-PCR) for selected transgenic plants. Primers (Cry160-For: 5'-GATTGGAAACTACACCGACC-3' and Cry160-Rev: 5'-GGAGTCATAGTTCGGGAAGA -3') amplifying 160 bp of the *cry1Ac* gene sequence were designed and used for the quantitative analysis of *cry1Ac* transgene expression in the transgenic plants. Primers (HMGIII-For: 5'-AGGGGTAGGCCGAAGAAGAT-3' and HMGIII-Rev: 5'-TGAGGCTTCACCTTAGGAGG -3', 164 bp) for a pea housekeeping gene (*HMG-I/Y*) (Gupta et al., 1997) as a reference were included in the analysis to normalize the expression of *cry1Ac* transgene.

The qRT-PCR was performed on iQ^{TM} 96-well PCR plates covered with Optical Sealing Tape (Bio-Rad) on iCycleriQ5 Real Time PCR detection system (Bio-Rad). The qRT-PCR mixture contained 3 pMol (0.3 µl from 10 pMol stock) of both forward and reverse primers, 7.5 µl of iQTM SYBR® Green Supermix (Bio-Rad) and 1 µl cDNA of each sample. The reaction volume was brought to 15 µl with sterile ddH_2O.

The qRT-PCR profile included:

- Cycle 1: Initial denaturation step of 95°C for 10 min,
- Cycle 2: 40 cycles of 95°C for 20 sec denaturation step, 59°C for 30 sec annealing step and 72°C for 45 sec extension step,

- Cyle 3: Denaturation step of 95°C for 60 sec
- Cylcle 4: Holding step of 55°C for 60 sec, and
- Cycle 5: Melting curve analysis steps of 55-95°C for 10 sec with 0.5 °C increment after each temperature point.

Each cDNA sample was duplicated three times. The qRT-PCR data were automatically collected and analyzed using iQ5 Optical System Software v2.0 (Bio-Rad). The unscaled normalized expression relative to the control was chosen in the gene expression analysis option.

3.3.2.3 Detection of Cry1Ac protein in transgenic pea plants

Commercially available immunostrip specific to Cry1Ab/Ac protein (Bt-Cry1Ab/1Ac Immunostrip® test, Agdia Inc.) was used for the detection of Cry1Ac protein in the progenies of transgenic pea lines. The extraction of crude protein and detection of the Cry1Ac protein was done according to the manufacturer's instruction. Briefly, 80-100 mg leaf sample was grinded in liquid nitrogen and 1500 µL extraction buffer (SEBA4, Agdia Inc.) was added and mixed using vortex. Then, 500 µL of the crude extract was transferred to a new 1.5 ml microcentrifuge tube and the immunostrip was inserted into the tube. Both control and test signals development was monitored on the immunostrip for a maximum of 30 min.

3.3.3 Leaf paint functional characterization of transgenic pea plants

Leaf paint functional characterization was used to characterize progenies of transgenic pea lines. The assay was conducted according to Schroeder et al. (1993) as described in Richter et al. (2006). Briefly, a Basta® herbicide solution (600 mg/L) with a drop of Tween20 was prepared and applied onto the upper side of the selected leaflet of both transgenic progenies and control plants while the opposite leaflet was marked as a control (Fig. 3). Then, the herbicide tolerance of the plants was evaluated one week after application.

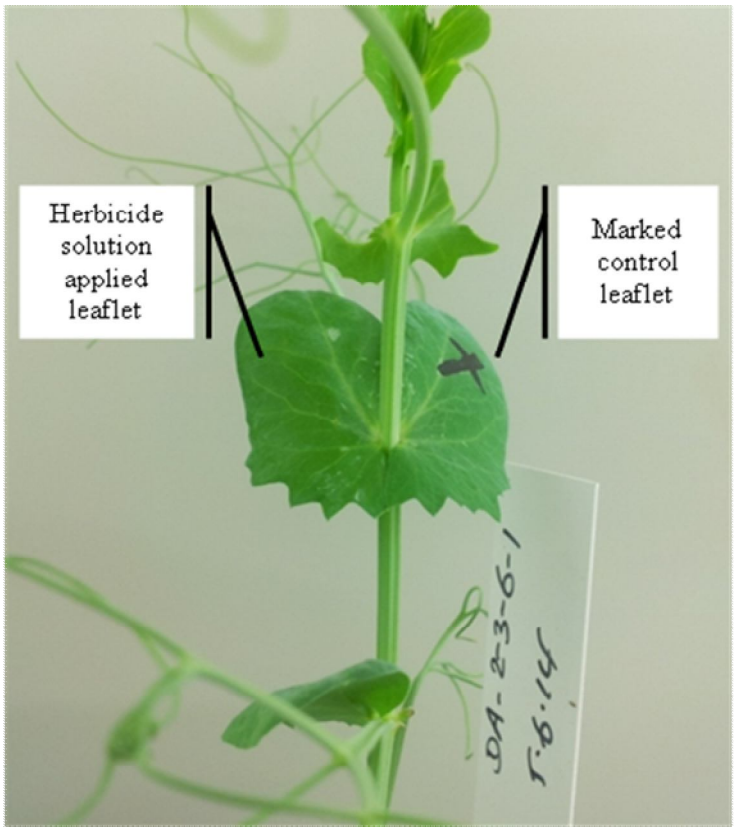


Fig. 3 Leaf paint functional characterization of transgenic plants

3.3.4 Insect bioassay

Seeds of both transgenic pea lines and control none-transgenic plants were grown in growth chamber (22±2 °C, 16/8 photoperiod). The transgenic pea plants were characterized for the presence of T-DNA region by PCR at DNA level, immunostrip assay at protein level and leaf paint assay at functional level. For some selected transgenic plants, the expression of *cry1Ac* gene at RNA level was also determined by RT-PCR and qRT-PCR.

Then, five larvae of *Heliothis virescens* (kindly provided by Dr. Jürgen Langewald, BASF Plant Science, Limburgerhof, Germany) were inoculated on each pea plant. The inoculated plants were covered with a glass cylinder to restrict the larvae movement (Fig. 4). Then, larval mortality and feeding damage were recorded on each plant one week after larvae inoculation.

Fig. 4 Larvae inoculated plants covered with glass cylinder to restrict larvae movement.

3.4 Results

3.4.1 PCR detection of the stable integration and inheritance of the transgene to the next generation

Putative transgenic shoots as well as the progenies of transgenic Cry1Ac pea lines were analyzed by PCR. Primers for *cry1Ac* and *bar* transgenes were used to specifically amplify the transgene segments. Primers for the *HMG*-I/Y housekeeping gene were included to monitor the presence of the genomic DNA during the amplification process. The result showed the presence of the transgene fragments in the genomic DNA of different transgenic lines (Fig. 5) confirming the stable integration of the transgene. The transgene was also inherited to the next generations for most of the analyzed transgenic lines. Up to T4-T7 advance generations were obtained for some of the transgenic lines (Appendix 4).

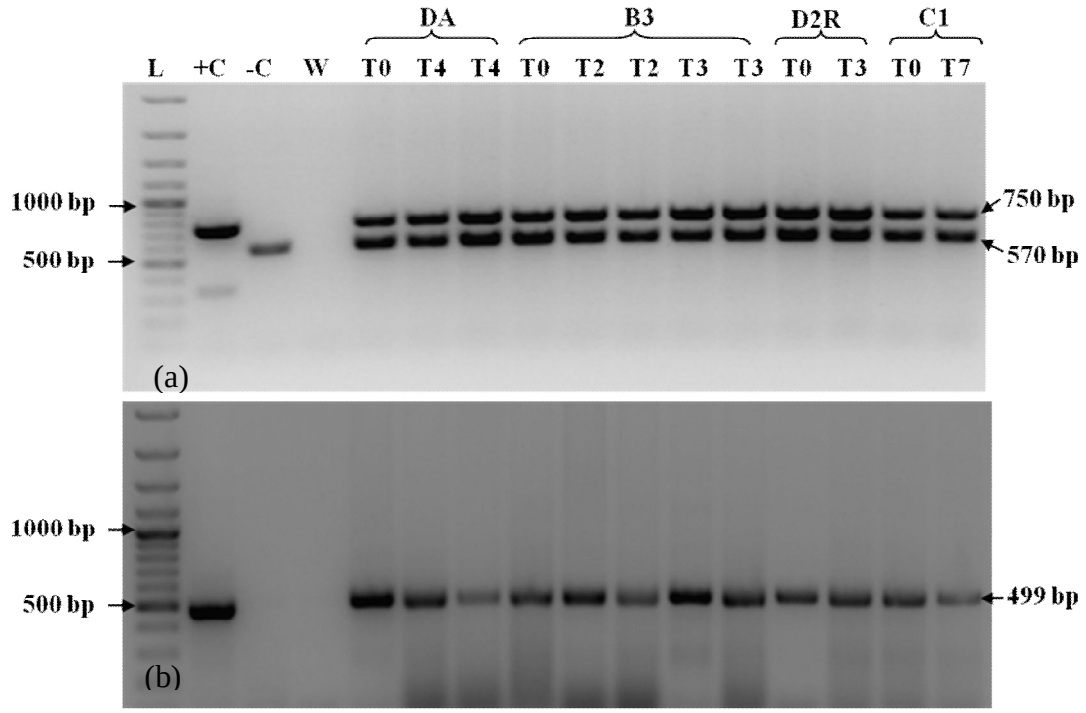


Fig. 5 PCR detection of transgene integration into the genome of few putative transgenic shoots and their advanced generations in Cry1Ac transgenic pea lines. (a) Putative transgenic shoots and their subsequent generation of transgenic pea lines analyzed using primers for *cry1Ac* (750 bp) and pea housekeeping (570 bp) genes indicating the stable genomic integration and inheritance of the *cry1Ac* transgene. (b) Putative transgenic shoots and their subsequent generation of transgenic pea lines analyzed using primers for *bar* gene. L: GeneRuler^{TM} 100 bp plus DNA ladder, +C: plasmid (pGII35S-cry1Ac) DNA as a positive control, -C: genomic DNA from non-transgenic pea plant as a negative control, W: water control; and DA, B3, D2R and C1 are different transgenic pea lines

3.4.2 RT-PCR detection of transgene expression in the transgenic pea plants

To analyze the expression of the *cry1Ac* gene at transcription level, the isolation of RNA and the synthesis of cDNA from selected PCR confirmed transgenic plants was done using the standard kits. The purity of the isolated RNA was checked with spectrophotometer by measuring the ratio of absorbance at 260:280 nm. The measured ratios ranged from 2.057-2.166 (Appendix 5). The integrity of the RNA was checked on agarose gel electrophoresis (Fig. 6a) indicating the intactness of the isolated RNA. There was no genomic DNA contamination in the isolated total RNA (Fig. 6b).

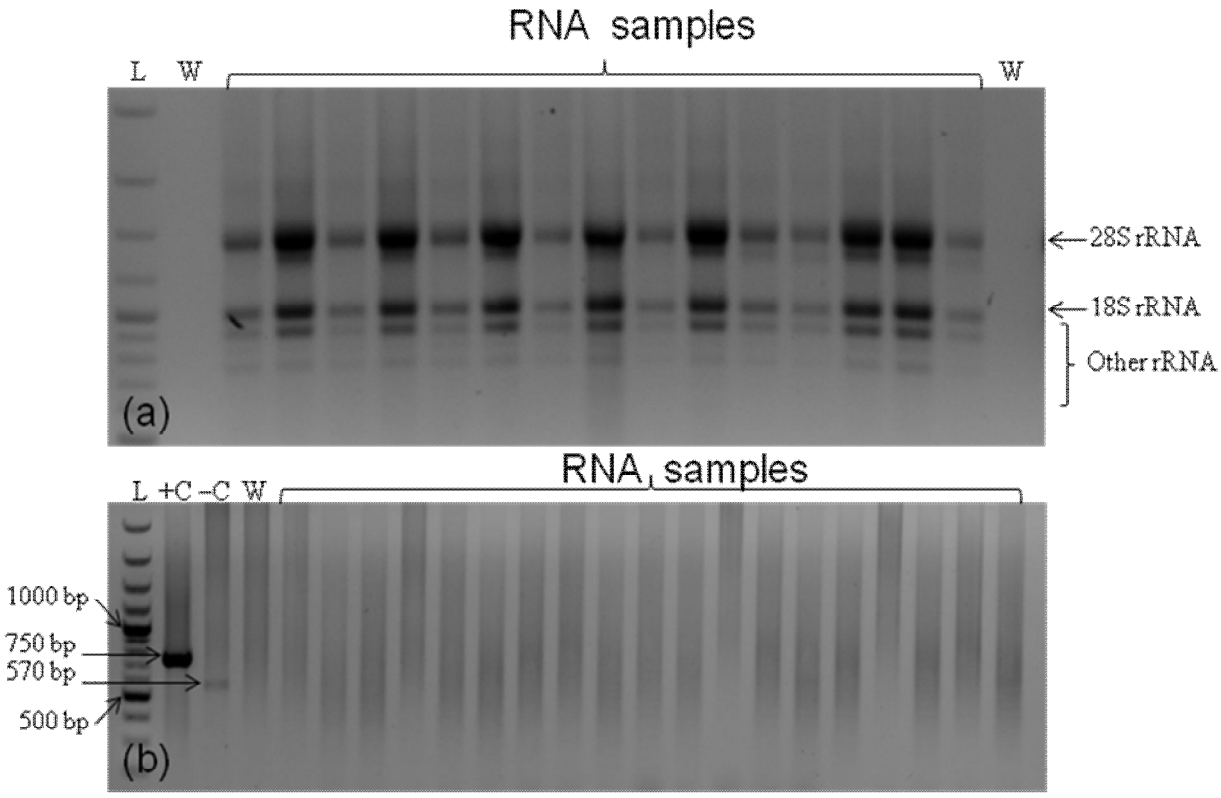


Fig. 6 Monitoring RNA integrity (a) on standard 1 % agarose gel and genomic DNA contamination (b) with PCR using primer for *cry1Ac* (750 bp) and pea *HMG-I/Y* (570 bp) genes. L: GeneRuler^{TM} 100 bp plus DNA ladder, +C: plasmid (pGII35S-cry1Ac) DNA as a positive control, -C: genomic DNA from non-transgenic pea plant as a negative control and W: water control.

The prepared cDNA was used in the PCR detection of the *cry1Ac* gene specific sequence. The result demonstrated the expression of the *cry1Ac* transgene at different progeny levels (T0 to T7) (Table 4). The expected PCR products for both *cry1Ac* and *HMG-I/Y* housekeeping genes were amplified in most of the analyzed plants (Fig. 7). Out of 58 T2-T7 generation plants analyzed from 15 transgenic lines, 44 plants from 13 transgenic lines showed the expression of *cry1Ac* gene at the transcriptional level. In the cDNA of the non-transgenic plant, as expected, only the sequence without intron for the HMG-I/Y housekeeping gene was amplified indicating the absence of genomic DNA in the synthesized cDNA.

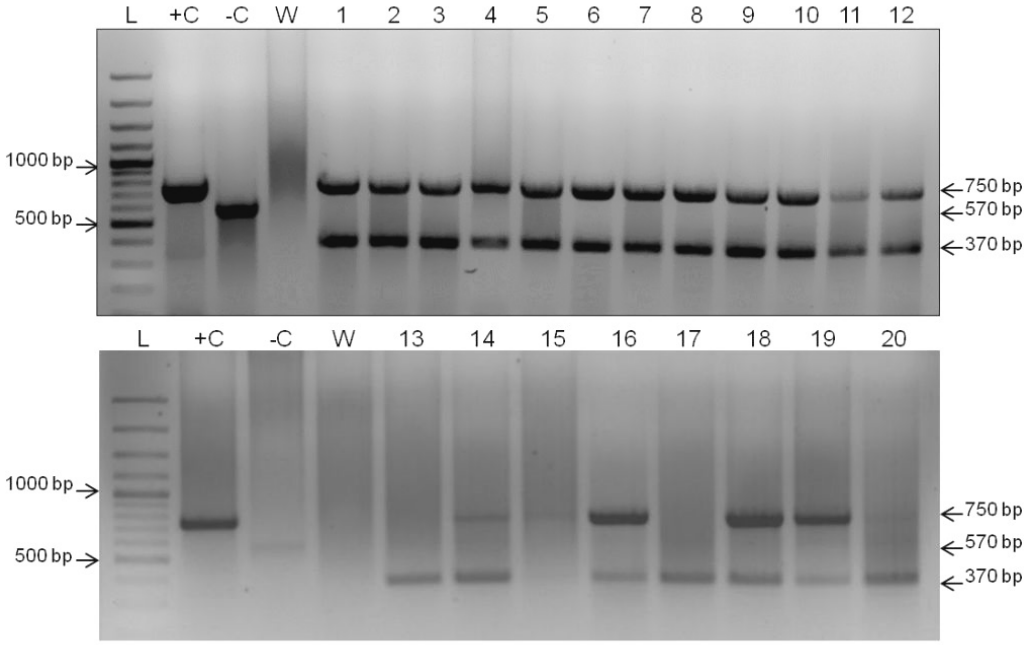


Fig. 7 RT-PCR expression analysis of *cry1Ac* transgene (750 bp) and pea *HMG-I/Y* housekeeping gene (570 bp from genomic DNA and 350 bp from cDNA) in the advanced progenies of different transgenic pea lines. L: GeneRuler^{TM} 100 bp plus DNA ladder, +C: plasmid (pGII35S-cry1Ac) DNA as a positive control, -C: genomic DNA of non-transgenic pea plant as a negative control; W: water control, lane 1-12 and 14-20: cDNA from different transgenic lines and lane 13: cDNA from non-transgenic control plant [1: D2R-1(T2), 2: E15(a)-2-1(T2), 3: G*A-1-1(T2), 4: BR-5-1-4-1(T4), 5: DA-2-3-6-1(T4), 6: DqR-8-7-2-1(T4), 7: C1-2-6-15-1(T4), 8: G51-2-2-3-1-1(T5), 9: C1-2-3-3-3-2-1(T6), 10: C1-2-6-13-1-3-6-1(T7),11: B1-2-1, 12: G51-1-5-1(T3), 14: B3-1-11-4-1(T4), 15: G51-1-11-1(T3), 16: A2/D12-1-1-1-1(T4), 17: A2R-2-1(T2), 18: G51-1-5-1 (T3), 19: D2R-2-12-1(T3) and 20: B1-2-1(T2)].

Table 4. Summary of RT-PCR expression analysis of *cry1Ac* gene in the transgenic pea lines

Plant code	**Progeny level**	**PCR for Cry1Ac**	**RT-PCR**	**Plant code**	**Progeny level**	**PCR for Cry1Ac**	**RT-PCR**
A3	T0	+	?[a]	B3-3-1-3	T3	-	
A2/D12	T0	+	+	B3-3-1-4	T3	+	+
B	T0	+	+	B3-3-1-5	T3	+	+
B2	T0	+	+	B3-3-2-1	T3	+	+
B2R	T0	+	+	BR-3-1	T2	+	+
B3	T0	+	+	BR-5-1	T2	+	+
BR	T0	+	+	BR-5-1-4-1	T4	+	+
BR*	T0	+	?	BR-5-2	T2	+	+
C7	T0	+	+	C1-1-2	T3	+	+
D	T0	+	+	C1-2-1-6-13-1	T5	+	+

[a]DNA contamination problem!

Table 4. Continuation

Plant code	Progeny level	PCR for Cry1Ac	RT-PCR	Plant code	Progeny level	PCR for Cry1Ac	RT-PCR
D1	T0	+	?	C1-2-1-6-13-2	T5	+	+
D2R	T0	+	+	C1-2-1-6-13-3	T5	+	+
D4R	T0	+	?	C1-2-1-6-13-5	T5	+	+
D21R	T0	+	?	C1-2-3-3-3	T4	+	+
D40	T0	+	?	C1-2-3-3-3-2-1	T6	+	+
DA	T0	+	+	C1-2-3-6-2	T5	+	+
DqR	T0	+	+	C1-2-6-13-1-3-6-1	T7	+	+
DR*	T0	+	+	C1-2-6-15-1	T5	+	+
DT	T0	+	+	C-5-1	T2	+	+
Danne	T0	+	?	C5-1-1	T3	+	-
Ddiff	T0	+	?	C5-2-1	T3	+	-
D20	T0	+	+	C5-2-2-1	T4	+	-
E	T0	+	+	C5-3-1	T3	+	-
E1	T0	+	?	C7-1-3-1	T3	+	-
E8	T0	+	?	C7-1-4-1	T3	+	-
E8R	T0	+	?	C7-1-4-3	T3	+	-
ER	T0	+	+	D2R-1	T2	+	+
G	T0	+	+	D2R-2-12-1	T3	+	+
G3	T0	+	+	D2R-2-9-5	T3	+	+
G4	T0	+	+	DA-2-1	T2	+	+
G*A	T0	+	?	DA-2-2	T2	+	-
GB	T0	+	+	DA-2-3	T2	+	+
GBR	T0	+	+	DA-2-3-6-1	T4	+	+
GqR'/GTR'	T0	+	?	DA-2-4	T4	+	+
A2/D12-1-1-1-1	T4	+	-	DqR-8-1	T3	+	+
A2/D12-1-3	T2	+	+	DqR-8-2	T3	+	+
A2/D12-1-4	T2	+	-	DqR-8-4	T3	+	+
A2R-2-1	T2	+	-	DqR-8-7-2-1	T4	+	+
B1-1-1	T2	+	+	DqR-8-8	T3	+	+
B1-1-2	T2	+	-	E1 5(a)-2-1	T2	+	+
B1-1-3	T2	+	-	G*A-1-1	T2	+	+
B1-2-1	T2	+	+	G51-1-11-1	T3	+	+
B3-1-11-4-1	T4	+	-	G51-1-5-1	T3	+	+
B3-1-4-2	T3	+	+	G51-2-2-3	T3	+	+
B3-3-1	T2	+	+	G51-2-2-3-1-1	T5	+	+
B3-3-1-1	T3	-		Positive Control (+)		-	+
B3-3-1-2	T3	+	+	Negative Control (-)		+	-

3.4.3 qRT-PCR analysis of *cry1Ac* transgene expression levels in the transgenic pea plants

The expression levels of the *cry1Ac* gene were determined using qRT-PCR for some of the transgenic lines. Primers amplifying the sequences of the *cry1Ac* and housekeeping genes at similar annealing temperature were designed and checked for the expected PCR products. Fig. 8 shows the amplification result for the primers indicating the expected PCR products for both primer sets.

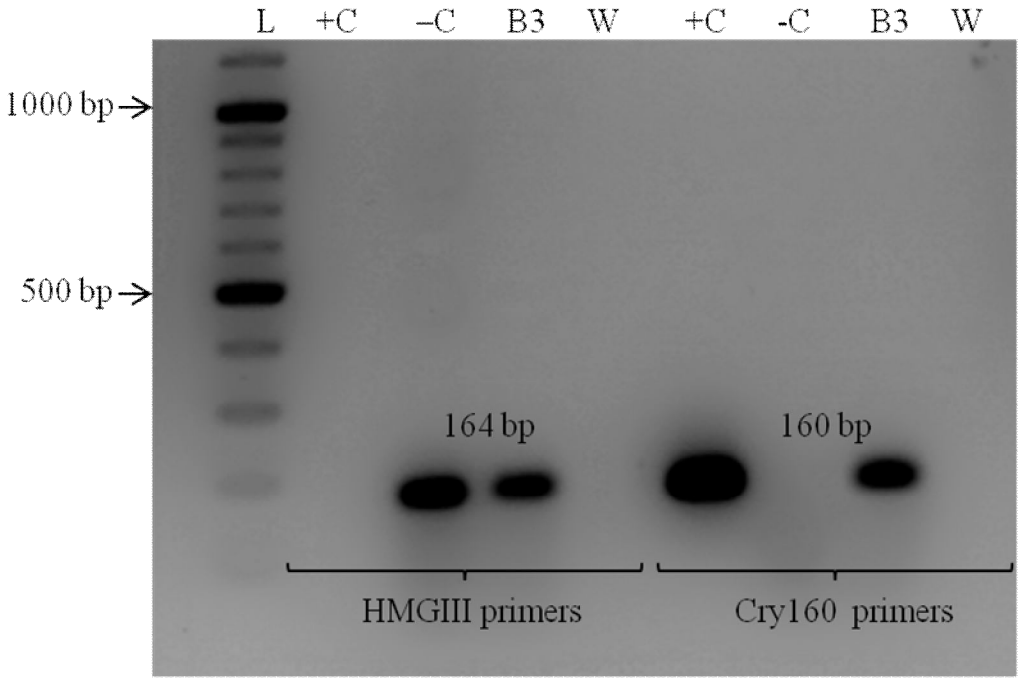


Fig. 8 Control PCR gel for primers used in qRT-PCR analysis indicating the expected PCR products for both housekeeping *HMG-I/Y* (164 bp) and *cry1Ac* (160 bp) genes. L: GeneRuler^{TM}100 bp plus DNA ladder, +C: plasmid (pGII35S-cry1Ac) DNA as a positive control, -C: genomic DNA of non-transgenic pea plant as a negative control; B3: genomic DNA from Cry1Ac transgenic pea plant and W: water control.

The designed primers were used for quantitative analysis of *cry1Ac* transgene expression normalized to the reference gene. PCR amplification (quantification) charts and the melting curve analysis for 18 transgenic plants are shown in Fig. 9. The PCR amplification charts indicate the relative fluorescence accumulation while the single peak on the melt curve analysis shows the presence of a single PCR product for each reaction tube.

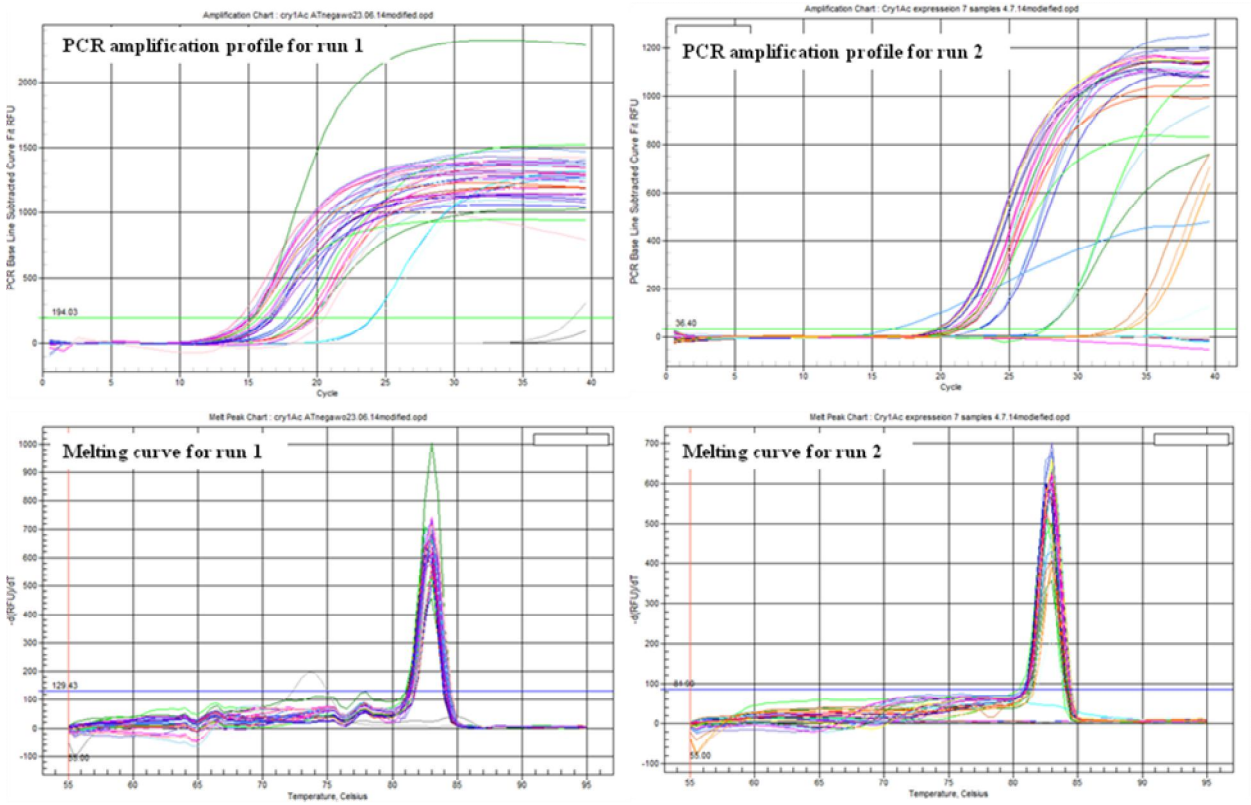


Fig. 9 PCR quantification profile (upper panel) and melting curve analysis (lower panel) of the amplifcation products produced during qRT-PCR analysis.The melting curve graphs show the negative derivative of fluorescence accumulation plotted against the temperature (°C) for each tube. The single peak for each line on the melting curve analysis shows the absence of unspecific PCR product for each reaction tube.

The normalized expression folds varied from line to line (Fig. 10). The expression levels ranged from very low (< 0.1) to 4.72 folds. Based on the expression folds, the transgenic plants were categorized to high (>1 folds), moderate (0.5-1 folds), low (0.1-0.499 folds) and very low (>0.1 folds) expression groups (Appendix 6). Eleven of the analyzed plants were grouped in high expression group. Of the plants in this group, the highest expression level (4.72 folds) was observed in A2/D12-1-1-1-1 plant followed by BR-5-1-4-1 (2.85 folds) and C1-2-6-13-1 (2.62 folds) plants. Another 11 plants showed low (7 plants) to moderate (4 plants) expression level. Eight plants showed very low expression level of the *cry1Ac* transgene.

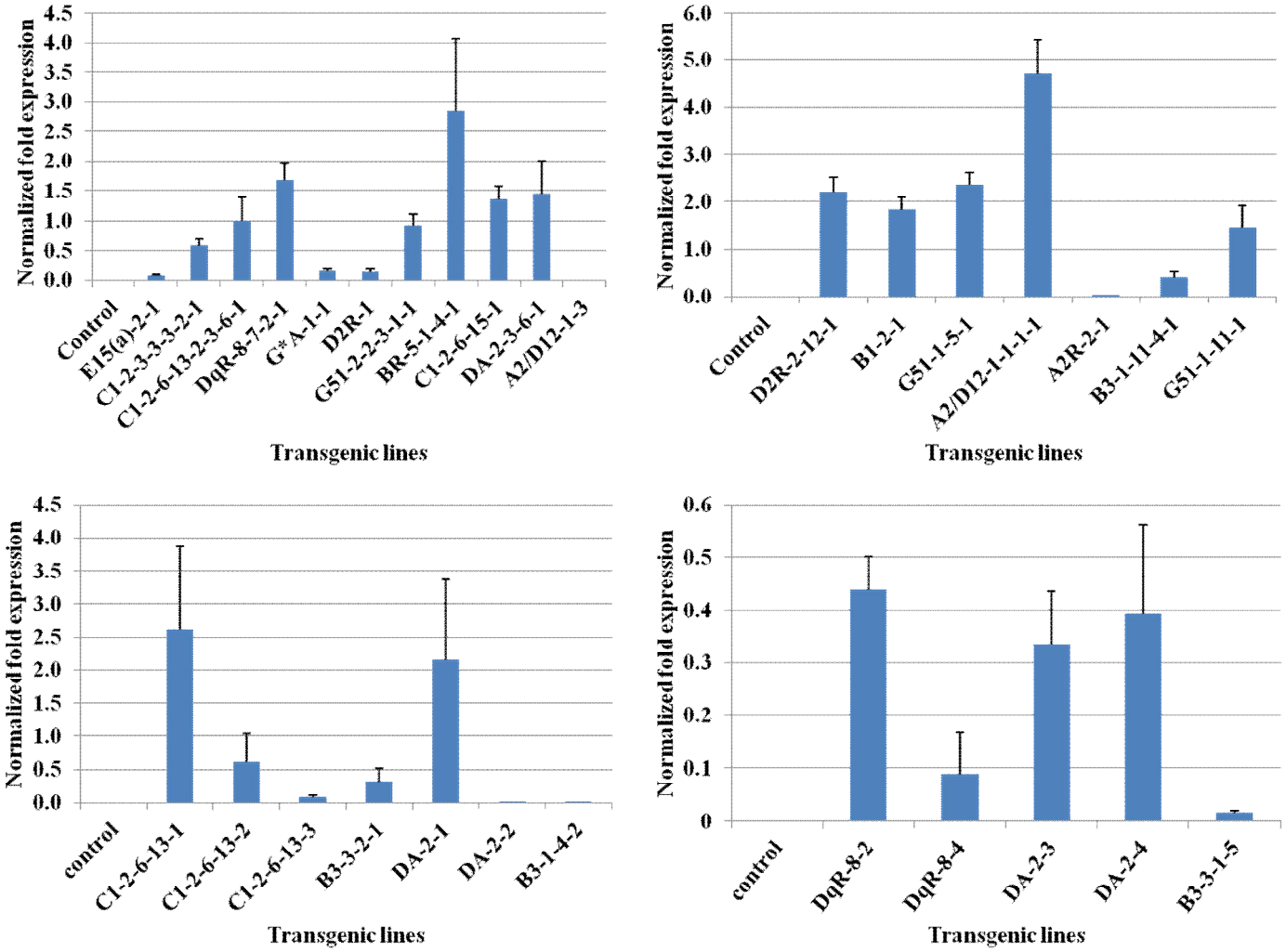


Fig. 10 Expression level of *cry1Ac* transgene in different transgenic pea lines. Normalized fold expression data represent the mean of three replicates (Mean ± SE). The analysis of plants indicated on the lower two graphs were done with the help of Linda Baranek.

3.4.4 Detection of Cry1Ac protein accumulation

The production of the Cry1Ac protein in the leaf tissue of transgenic progenies of the Cry1Ac pea lines was detected using a commercial detection strip for Cry1Ab/Cry1Ac protein. The detection was done using crude protein extracted from leaves of both non-transgenic control and PCR positive transgenic plants. In the protein extract of all tested plants (both non-transgenic control and transgenic plants), the expected control signal developed on the immunostrip. The expected test signal for the Cry1Ac protein was observed in the protein extract of most of the transgenic plants. On the other hand, the test signal was not developed on the immunostrip placed in the protein extract of control and few of the tested transgenic plants. Fig.11 shows the immunostrip result for a crude protein extracted from control and transgenic plants and Table 5 shows summary of the immunostrip detection of Cry1Ac protein for all the tested transgenic plants.

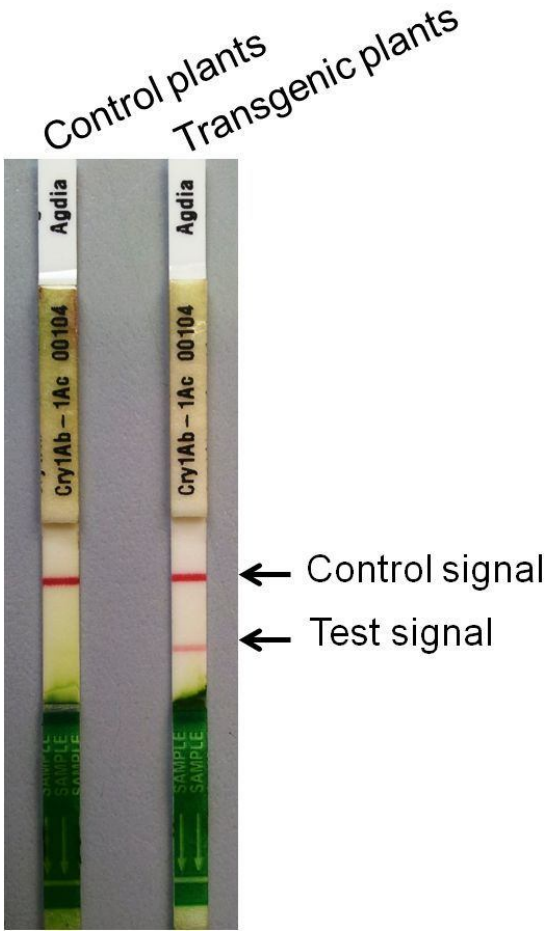


Fig. 11 Immunostrip detection of Cry1Ac protein in control and transgenic pea plants.

Table 5. Summary of Immunostrip detection of Cry1Ac protein in the progenies of Cry1Ac pea plants

No.	Transgenic line	Progeny level	Immunostrip signal		No.	Transgenic line	Progeny level	Immunostrip signal	
			Control	Test				Control	Test
1	Control	-	+	-	19	DqR-8-4-5	T3	+	+
2	A2/D12-1-1	T2	+	+	20	DqR-8-4-6	T3	+	-
3	B1-2-1	T2	+	-	21	BR-5-1-1	T3	+	+
4	E1 5(a)-2-1	T2	+	+	22	BR-5-1-4	T3	+	+
5	G*A-1-1	T2	+	+	23	DA-2-3-6	T3	+	+
6	B3-1-11-4-1	T4	+	-	24	DA-2-3-9	T3	+	+
7	D2R-2-12-1	T3	+	+	25	DqR-8-7-1	T3	+	+
8	G51-1-11-1	T3	+	+	26	DqR-8-7-2	T3	+	+
9	DA-2-1-1	T3	+	+	27	A2/D12-1-1-1	T3	+	+
10	DA-2-1-2	T3	+	+	28	G51-2-2-3-1	T3	+	+
11	DA-2-1-3	T3	+	-	29	G51-2-2-3	T3	+	+
12	DA-2-1-4	T3	+	+	30	B3-1-4-6	T3	+	+
13	DA-2-1-5	T3	+	-	31	A2/D12-1-1-1-1	T4	+	+
14	DA-2-1-6	T3	+	-	32	BR-5-1-4-1	T4	+	+
15	DqR-8-4-1	T3	+	+	33	C5-2-2-1	T4	+	+
16	DqR-8-4-2	T3	+	+	34	DA-2-1-1-1	T4	+	+
17	DqR-8-4-3	T3	+	+	35	DA-2-1-4-1	T4	+	+
18	DqR-8-4-4	T3	+	+	36	DA-2-3-6-1	T4	+	+

Table 5. Continuation

No.	Transgenic line*	Progeny level	Immunostrip signal		No.	Transgenic line	Progeny level	Immunostrip signal	
			Control	Test				Control	Test
37	DA-2-3-9-1	T4	+	+	54	T3 B3-3-1-2	T3	+	-
38	DqR-8-7-2-1	T4	+	+	55	T3 B3-3-1-3	T3	+	-
39	C1-2-3-6-2	T4	+	+	56	T3 B3-3-1-4	T3	+	-
40	C1-2-6-15-1	T5	+	+	57	T3 B3-3-1-5	T3	+	-
41	G51-2-2-3-1-1	T5	+	+	58	T3 B3-1-4-2	T3	+	-
42	C1-2-3-3-3-1	T5	+	+	59	T3 B3-1-4-4	T3	+	-
43	C1-2-3-3-3-2	T5	+	+	60	T3 B3-1-4-5	T3	+	-
44	C1-2-3-3-3-3	T5	+	+	61	T3 B3-1-4-6	T3	+	+
45	C1-2-1-6-13-1-1	T5	+	-	62	T2 DA-2-2	T2	+	-
46	C1-2-1-6-13-1-2	T5	+	+	63	T1 DqR-8-6	T1	+	-
47	C1-2-3-3-3-2-1	T6	+	+	64	T1 DqR-8-7	T1	+	+
48	C1-2-1-6-13-1-3-1	T6	+	+	65	T5 C1-2-3-3-3	T5	+	+
49	C1-2-1-6-13-1-3-6	T6	+	+	66	T3 A2/D12-1-1	T3	+	+
50	C1-2-1-6-13-1-3-6-1	T7	+	+	67	T2 BR-5-1	T2	+	+
51	T3 B3-3-2-1	**T3**	**+**	**-**	68	T3 C1-1-2	T3	+	-
52	T3 B3-3-2-4	**T3**	**+**	**-**	69	T4 G51-2-2-3	T4	+	+
53	T3 B3-3-1-1	**T3**	**+**	**-**	70	T3 DR21R-2-2	T3	+	-

*Plant number from 51-70 was done with the help of Linda Baranek.

3.4.5 Leaf paint functional characterization of progenies of Cry1Ac transgenic pea lines

Fig. 12 shows the result of the leaf paint assay for some of the plants. In the progenies of transgenic pea lines, both herbicide tolerant (complete or partial) and susceptible plants were observed (Fig. 12 a-h, Appendix 4). The leaves of all control plants were susceptible to the applied herbicide solution (Fig. 12, i). The result demonstrated the functionality of the *bar* gene product in the developed transgenic lines.

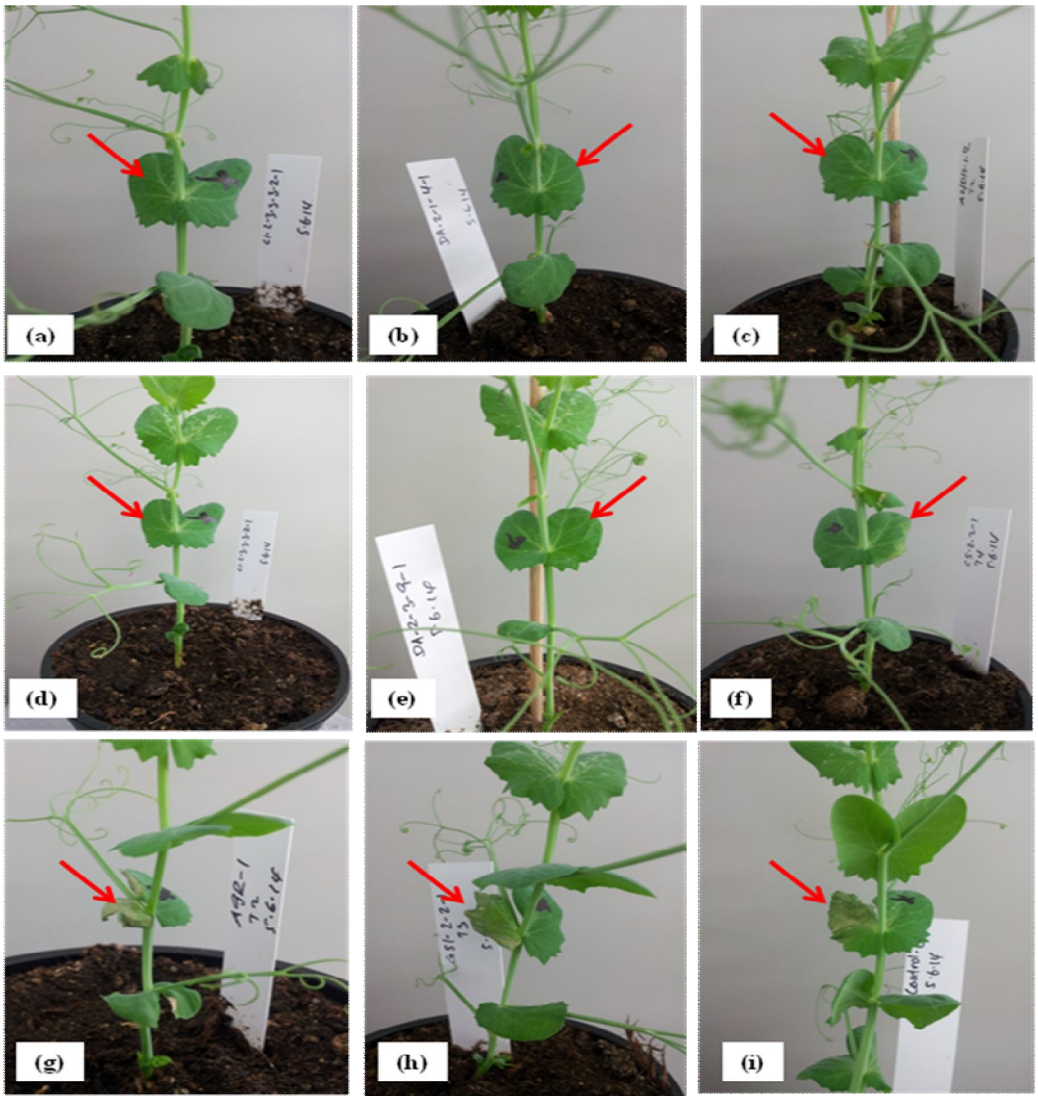


Fig. 12 Leaf paint functional assay result for some of the assayed plants showing herbicide tolerant (a-e: complete and f: partial) and susceptible (g-h) transgenic plants from different transgenic lines and herbicide susceptible non-transgenic control plant (i). The red arrows indicate the herbicide solution treated leaves.

3.4.6 Evaluation of insect resistance of the Cry1Ac transgenic pea plants

3.4.6.1 Larval mortality on Cry1Ac transgenic pea plants

Progenies from different transgenic lines were subjected to insect bioassays. Table 6 shows the result of larval mortality studies on the different transgenic lines and Fig. 13 shows some of the dead and surviving larvae collected at the end of the assay period from transgenic and control plants, respectively. Total larval mortality was recorded on five of the transgenic lines. Of the tested lines, four lines showed about 73-92 % larval mortality while three lines showed less than 20 % larval mortality. On one transgenic line (C7), the recorded mortality rate was less than that observed on control plants (17.42 %).

Table 6. Larva mortality on different transgenic pea lines

Plant line	Progeny level used	Number of plants[+]	Number of larvae			Mortality rate (%)	Corrected mortality rate (%)[a]
			Inoculated	Survived	Died		
Control plants		31	155	128	27	17.42	
Transgenic lines							
A2/D12-1 line	T2-T4	4	20	2	18	90	87.89
A2R-2-1	T2	1	5	4	1	20	3.13
B1-line	T2	2	10	7	3	30	15.23
B20R-2-1	T2	1	5	2	3	60	51.56
B3-line	T3-T4	14	70	38	32	45.71	34.26
BR-5-line	T4	2	10	0	10	100	100.00
C1-line	T3-T7	10	50	11	39	78	73.36
C5-line	T2-T4	4	20	15	5	25	9.18
C7-line	T2-T3	2	10	9	1	10	-8.98
C8R-line	T2	1	5	2	3	60	51.56
D2R-line	T2-T3	2	10	0	10	100	100.00
DA-line	T2-T4	10	50	6	44	88	85.47
DqR-line	T2-T4	6	30	2	28	93.33	91.93
E15-line	T2	1	5	0	5	100	100.00
G*A-line	T2	1	5	0	5	100	100.00
G51-line	T3-T5	7	35	0	35	100	100.00

[+]Transgenic progenies from the same line were pooled together. [a]Corrected mortality is calculated using the following formula as described in Jia (Jia, 2004): Corrected Mortality rate on transgenic plants (%)= [(LMRT-LMRC)/(100-MRC)] x 100; Where LMRT: Larva Mortality rate (%) on transgenic plants and LMRC: Larva mortality rate (%) on control plants.

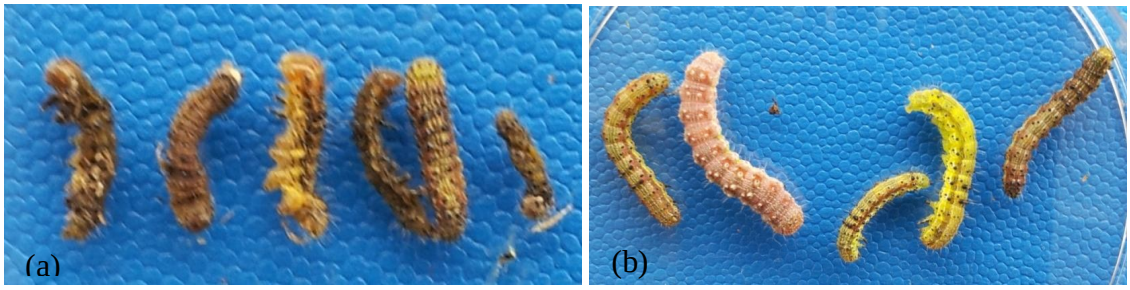


Fig. 13 The physical appearances of few of the dead larvae (a) after feeding on the transgenic plants and surviving larvae (b) collected from control plants at the end of the feeding test.

Fig. 14 shows the larval mortality and survival on individual transgenic plants for which the expression level of the *cry1Ac* gene was determined by qRT-PCR. Total larval mortality was recorded on transgenic plants expressing varying level of the *cry1Ac* gene. On most the transgenic plants in the high expression group and some of the plants in other expression groups (low to moderate such as G*A-1-1, D2R-1, DqR-8-4, etc), total larval mortality was recorded. On other transgenic plants (For example: C1-2-6-13-2-3-6-1, B1-2-1, C1-2-6-13-3, C1-2-6-13-2, etc), in which the expression folds of the *cry1Ac* gene was moderate to high, 2-3 larvae died on each plant. On one plant (A2R-2-1) in the low expression group, only one larva died compared to 2-5 larvae on other plants.

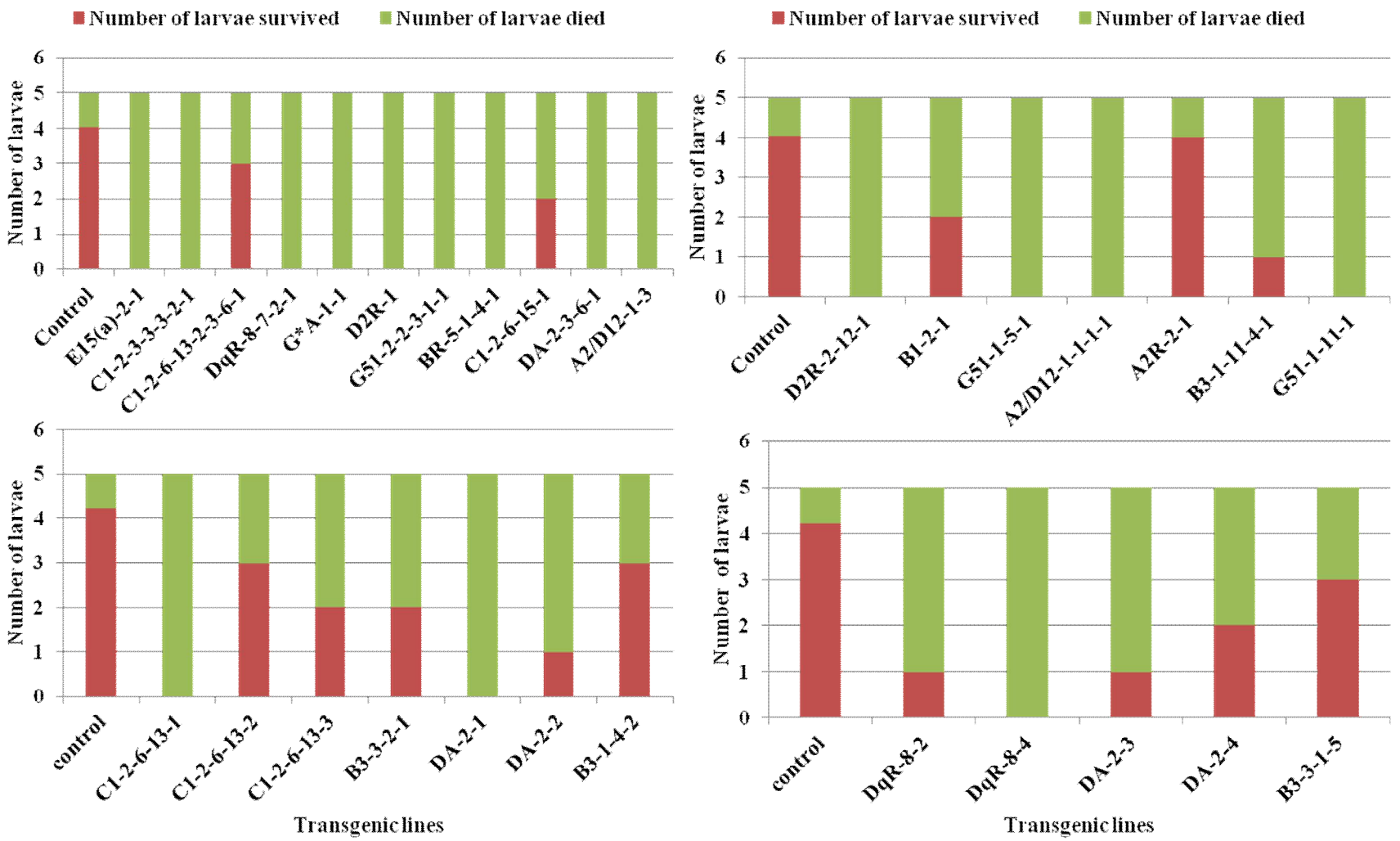


Fig. 14 Larval mortality and survival on individaul plants of different transgenic lines expressing *cry1Ac* gene. The experiment for the lower two graphs were done with the help of Linda Baranek.

3.4.6.2 Larval feeding damage on Cry1Ac transgenic pea plants

Similar to larva survival, the average feeding damage on transgenic plants varied from line to line (Table 7). Fig. 15 shows relative feeding damage on one of the control and transgenic plants.

On the transgenic plants, the average feeding damage ranged from very negligible (recorded as 5 %) to 57.50 %. On plants from nine transgenic lines, the estimated feeding damage was about 5-11 percent. More than 50 % average feeding damage was recorded on plants from two transgenic lines. Among the transgenic plants, the maximum feeding damage was observed on the transgenic plants from the C7-line. On control plants, the average feeding damage caused by the inoculated larvae was about 53.06 %. More than 70 % (22 out of 31) of the control plants incurred 50 % or more feeding damage. In the transgenic lines, nine plants from four transgenic lines incurred 50 % or more feeding damage.

Table 7. Estimated feeding damage on different transgenic lines

Plant line	Progeny level used	Number of plants[+]	Number of plants with estimated feeding damage of				Average estimated feeding damage (%)
			0-10%	11-20%	21-49%	50-100 %	
Control plants		31	1	1	7	22	53.06
Transgenic lines							
• A2/D12-1 line	T2-T4	4	4	0	0	0	5.00
• A2R-2-1	T2	1	0	0	1	0	35.00
• B1-line	T2	2	0	0	1	1	55.00
• B20R-2-1	T2	1	0	0	1	0	25.00
• B3-line	T3-T4	14	2	6	1	5	28.93
• BR-5-line	T4	2	2	0	0	0	5.00
• C1-line	T3-T7	10	6	3	1	0	11.50
• C5-line	T2-T4	4	0	1	1	2	46.25
• C7-line	T2-T3	2	0	1	0	1	57.50
• D2R-line	T2-T3	2	2	0	0	0	5.00
• DA-line	T2-T4	10	10	0	0	0	5.50
• DqR-line	T2-T4	6	6	0	0	0	5.00
• E15-line	T2	1	1	0	0	0	5.00
• G*A-line	T2	1	1	0	0	0	5.00
• G51-line	T3-T5	7	7	0	0	0	5.00

[+]Transgenic progenies from the same line were pooled together.

Fig. 15 The state of (a) non-transgenic control plant and (b) transgenic plant after one week of larvae feeding (Negawo et al., 2013).

Fig. 16 shows the recorded feeding damage on the different transgenic plants for which the expression level of the *cry1Ac* gene was determined by qRT-PCR. On most of the transgenic plants, despite varying level of expression folds, the estimated feeding damage was very negligible (~5 %) compared 40 and 60 % estimated average feeding damage on control plants. Relatively higher feeding damage of 15-25 % was recorded on few of the transgenic plants (C1-2-6-15-1, C1-2-6-13-2-3-6-1, B1-2-1, C1-2-6-13-2, B3-3-2-1) grouped in moderate to high expression groups. On transgenic plants A2R-2-1 and B3-3-1-5 (both with very low expression folds), the estimated feeding damage was 35 and 50 %, respectively.

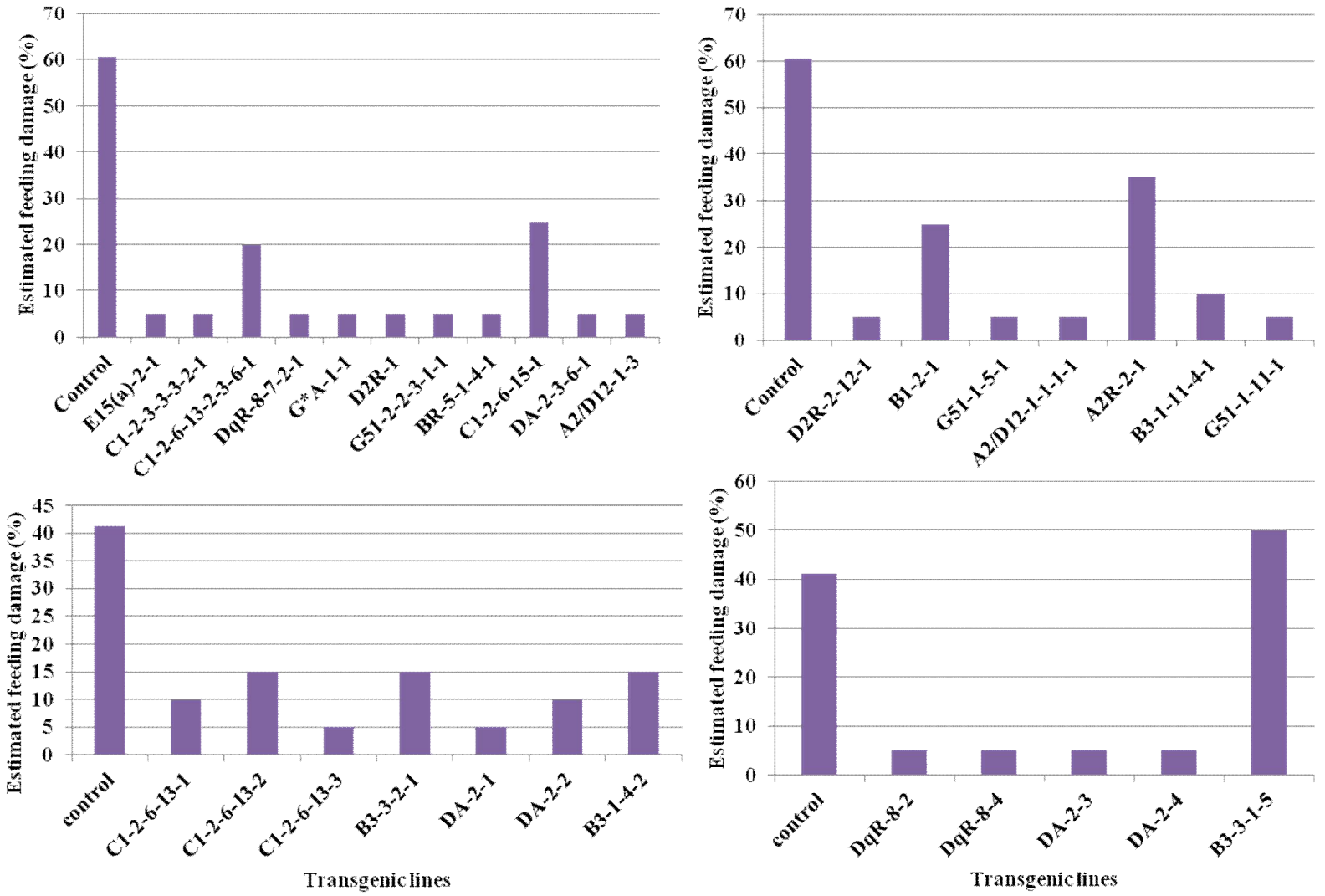


Fig. 16. Feeding damage on individaul plants of different trasgenic lines expressing the *cry1Ac* gene. The experiment for the lower two graphs were done with the help of Linda Baranek.

3.5 Discussion

Pea has been manipulated *in vitro* since more than three decades. These long term efforts have resulted in vital information about the *in vitro* regeneration and transformation of pea. Today, pea is one of the crop plants which have a routine regeneration and transformation protocol based on *Agrobacterium*-mediated transformation. The Cry1Ac transgenic pea lines (Negawo, 2012) used in this study are also the result from the application of such established protocol.

3.5.1 Inheritance and expression of *cry1Ac* gene

Progenies of different Cry1Ac transgenic pea lines have been characterized using molecular and functional analyses. The results of molecular analyses (such as PCR, RT-PCR and immunostrip assay) indicated the stable integration, inheritance as well as the expression of the introduced T-DNA region (*cry1Ac* and *bar* genes). The result of leaf paint assay also indicated the presence and functionality of the *bar* gene product. This assay has been used by different groups (Richter et al., 2006; Hassan et al., 2009) to characterize progeny of transgenic pea plants obtained after transformation with transformation vector harboring *bar* gene. They reported that transgenic progeny which inherited and expressed the *bar* gene was resistant to the herbicide application while others are not. In the current analysis, similar result was observed. In the progeny of transgenic lines, both herbicide tolerant and susceptible plants were observed as expected in segregating materials. This result further supports the molecular results and confirms the presence/inheritance as well as the functional expression of the T-DNA region in the developed transgenic lines.

Attempts have also been made to analyze the segregation ratio of the transgene for some of the transgenic pea lines (Appendix 7). However, the number of seeds per line was very few which could either over or under estimated the result. Hence, the data were only used to track the inheritance of the transgene to the next generation.

The use of transgenic plants depends on the faithful inheritance and expression of the novel traits (Finnegan and Mcelroy, 1994; Meyer, 1995). Despite the genomic integration, the expression of transgene could vary from line to line and/or could be silenced at all (Meyer,

1995; Kohli et al., 2006; Kohli et al., 2010). As reviewed by different authors (Iyer et al., 2000; Kohli et al., 2006; Kohli et al., 2010), variation in transgene expression has been reported in many transgenic plants such as petunia, tobacco, rice, wheat and maize. Similarly, variation in expression folds of *cry1Ac* gene was also observed in the analyzed transgenic pea lines. In the progeny of few transgenic lines (such as C7-1-3-1, C7-1-4-1, C5-1-1 and C5-3-1), despite the integration and inheritance of the *cry1Ac* gene was demonstrated by PCR, the expression of *cry1Ac* gene was not observed at transcription level (RT-PCR result). This is also supported by the negative result of immunostrip assay for Cry1Ac protein and/or leaf paint assay for *bar* gene product. It could be possible the introduced T-DNA region has been silenced in these transgenic plants. In transgenic plants, transgene silencing is a common phenomenon which can be due to transgene copy number, integration pattern, integration region and/or epigenetic effect and can occur at transcription and/or post-transcription level (Finnegan and Mcelroy, 1994; Stam et al., 1997; Kohli et al., 2010). For example, in transgenic clone B3 and its progenies, the result from Southern blot analysis showed the presence of multiple copies of the transgene (Negawo, 2012). Most of the progenies from this line showed negative result for the immunostrip and LP assay. Hence, without ruling out other factors, it might be possible that the copy number has contributed to the silencing of the T-DNA in these plants. Transgene integration is a random process in which the transgene is inserted anywhere in the host genome (Chyi et al., 1986; Wallroth et al., 1986; Kohli et al., 2006) with consequence on the expression level (Stam et al., 1997; Kohli et al., 2006; Kohli et al., 2010). In petunia, the integration of transgene in the highly repetitive DNA and methylated region of the genome has caused inactivation of the integrated transgene (Prols and Meyer, 1992).

3.5.2 Insect resistance evaluation of the *Cry1Ac* transgenic pea lines

In support of the molecular and leaf paint functional analyses, the insect bioassay results have demonstrated the functionality of Cry1Ac protein in some of the analyzed transgenic lines. Up to 100 % larval mortality as well as substantially reduced plant damage was observed on the transgenic plants expressing the transgene indicating the insect resistance of the developed transgenic lines. On the other hand, on transgenic plants in which the T-DNA region seemed to be silenced (negative RT-PCR and LP results) or expressed at very low level (qRT-PCR result), the insect bioassay showed the susceptibility of the plants to the

larval challenge. For example, in transgenic plants A2R-2-1 and B3-3-1-5 the result of qRT-PCR showed very low expression of *cry1Ac* gene. Similarly, on transgenic plants C7-1-4-1, C7-1-4-2, B1-1-1, B3-3-1-5 and A2R-2-1, the LP assay showed susceptibility to the applied herbicide solution. On these plants, less larval mortality rate (1-2 out of 5 larvae) and relatively high feeding damage (29-57.5 %) was recorded.

Similar results have been reported on other transgenic crops expressing *cry1Ac* or other *cry* genes. Reduced feeding rate to complete larval mortality of *Manduca sexta* was reported on transgenic tobacco plants expressing *B.t* crystal proteins (Barton et al., 1987; Vaeck et al., 1987). More recently, complete mortality of larvae of two insect pests (*Manduca sexta* and *Heliothis virescens*) and negligible leaf damage was observed on transgenic tobacco plants expressing *cry1Ac* gene under the control of wound-inducible promoter (Gulbitti-Onarici et al., 2009). Up to 100 % larval mortality of Lepidopteran insect pests (*Manduca sexta, Heliothis virescens, Helicoverpa armigera and Spodoptera litura*) and very little sign of feeding damage has been reported on transgenic tomato plants expressing crystal toxin from *B.t* var. Kurstaki HD-1(Fischhoff et al., 1987) and Cry1Ab protein (Koul et al., 2014). Total larval mortality of two insect pests (cabbage looper and beet armyworm) and high level of plant protection have been observed on transgenic cotton plants expressing *cry1Ac* or *Cry1Ab* genes from *B.t* var. Kurstaki (HD-1 and HG-73, respectively) (Perlak et al., 1990). Different cry toxins (*cry1Ab, cry1ac* or *cry1B)* have also been expressed in cereal crops like rice (Cheng et al., 1998; Datta et al., 1998; Breitler et al., 2001; Khanna and Raina, 2002; Ramesh et al., 2004), maize (Koziel et al., 1993; Armstrong et al., 1995; Bohorova et al., 1999) and sorghum (Girijashankar et al., 2005). According to the reports, up to 100 % larval mortality of the target insect pests as well as substantially reduced plant damage has been observed on the transgenic plants of these crops.

Similarly, in few legume crops, transgenic insect resistance has been pursued during the last decades. *B.t cry* gene (*cry1Ac, cry1Ab,* etc) has been introduced into the genome of chickpea (Kar et al., 1997; Sanyal et al., 2005; Indurker et al., 2007; Acharjee et al., 2010; Biradar et al., 2010; Indurker et al., 2010; Mehrotra et al., 2011), cowpea (Adesoye et al., 2008; Bakshi et al., 2011; Bakshi and Sahoo, 2013), peanut (Singsit et al., 1997) and soybean (Parrott et al., 1994; Stewart Jr et al., 1996) to develop resistant lines to the larvae of Lepidopteran insect pests. Different level of resistance to the target insect pests has been achieved in few of these legumes. However, despite the reports, follow up works on the claimed transgenic lines are

lacking in most of the cases. In legume crops, transgenic approaches have also been used to develop resistance to storage pests. For this purpose, bean alpha amylase inhibitor has been introduced into the genome of pea, cowpea and chickpea, and high level of resistance to the target storage pests (weevils) was achieved (Shade et al., 1994; Sarmah et al., 2004; Solleti et al., 2008a). However, the development of transgenic insect resistance in pea using *B.t cry* genes has not been given due attention during the last decades. Hence, this study has contributed to the development of Cry1Ac transgenic pea lines by characterizing different transgenic lines at molecular and functional levels.

3.5.3 Summary and future outlook

In general, Cry1Ac transgenic pea plants have been characterized at molecular and functional levels and the result demonstrated the successful development of insect resistant Cry1Ac transgenic pea lines.

The scope of this study was limited to laboratory and greenhouse experiments. Field trial was not conducted on the developed transgenic lines. Hence, further research activities are required to evaluate the field performance of the transgenic lines under natural growing conditions. Then, selected transgenic lines could be used in production program to reduce yield losses due to the target pest or in improvement program to combine with other traits for multiple resistances.

4. Regeneration and *Agrobacterium*-mediated transformation of cowpea

Alemayehu Teressa Negawo[1], Fathi Hassan[1] and Hans-Jörg Jacobsen[1*]

[1]*Gottfried Wilhelm Leibniz Universität Hannover, Institute for Plant Genetics, Section of Plant Biotechnology, Herrenhäuserstr.2, 30419 Hannover, Germany.*

**Correspondence address*: Jacobsen@genetik.uni-hannover.de

4.1 Abstract

Cowpea is susceptible to many insect pests during its life cycle. Thus, genetic transformation can be used to introduce genes for insect resistance into the variety of interest. Hence, in this study, different regeneration and transformation conditions were optimized. Transformation vectors containing *GUS* and *GFP* genes were used for transient transformation studies.

Cowpea has been regenerated from cotyledonary node (CN) explants on a medium containing varying level of BA alone or in combination with 0.5 µM Kinetin or NAA. Medium supplemented with 3 µM BA and 0.5 µM Kin was optimal for multiple shoot production. CN explants obtained from BA supplemented pre-conditioning medium showed better shoot production rate than those from medium supplemented with TDZ. Rooting of *in vitro* shoots was obtained on media with or without IBA, and the *in vitro* rooted plantlets were successfully acclimatized and transferred to greenhouse. Based on the transient transformation, embryo explants from dry seeds showed better transformation efficiency as compared to CN explants. Transformation efficiency was improved by adding 200 µM acetosyringone and 1 mM Na-thiosulphate to the inoculation/co-cultivation medium. In addition, explant orientation during co-cultivation was also indicated as one of the *in vitro* factors affecting transformation in terms of the area of explant expressing the *GUS* gene. Based on the optimized protocol, a series of transformation experiment were conducted to introduce B.t *cry* genes into the genome of cowpea plants. PCR analysis of putative transgenic shoots showed presence of *cry1Ac* gene in the genomic DNA from two *in vitro* shoots of one experiment while positive result was not achieved in the rest of the

transformation experiments. RT-PCR and immunostrip analyses of the primary transformants showed the expression of the *cry1Ac* gene at RNA and protein levels, respectively. Hence, given the recalcitrance of the crop, further works are required to explore the *in vitro* conditions for successful transformation of cowpea in the future.

Key words: cowpea, regeneration, transformation, Agrobacterium, transgenic approach, insect pests, recalcitrance

4.2 Introduction

Cowpea (*Vigna unguiculata* L. Walp., 2n=2x=22) is a multipurpose legume widely grown in Africa, Asia and Latin America (Brar et al., 1997a; Brar et al., 1997b; Ehlers and Hall, 1997; Timko and Singh, 2008). It is used as a food for human consumption and feed for animals (Timko and Singh, 2008). Cowpea contains high protein level that makes it a good and cheap source of dietary protein for millions of people mainly in Africa and Asia (Brar et al., 1997b; Diouf and Hilu, 2005).

The production of cowpea is affected by many biotic and abiotic factors. Insect pests are one of the biotic factors causing substantial yield losses by attacking cowpea both in the field and during storage (Jackai and Daoust, 1986). There is always a major pest that could cause damage to the plants and grains in the life cycle of cowpea (Fatokun, 2002). Some of the most important pests include cowpea pod borer (*Maruca vitrata),* cowpea seed moth (*Cydia ptychora*), cowpea weevil (*Callosobruchus maculatus*), cowpea leaf beetle (*Ootheca mutabilis*) and cowpea curculio (*Chalcodermus aeneus*) (Jackai and Daoust, 1986; Popelka et al., 2004; Chaudhury et al., 2007).

Benefits of genetic transformation in crop improvement have been well documented. With the application of genetic transformation, there is no need to expect cross compatibility to transfer trait from one species to another. This has opened opportunities to use gene of interest for new trait from any source (Korth, 2008). Cross incompatibility is one of the challenges breeders and researchers have managed to overcome through genetic transformation. In some crops, some of the economically important traits are lacking or available in distant relative species which makes the application of conventional improvement approaches very difficult or sometimes impossible. For example, resistance trait

for insect pests is lacking in cowpea (Chaudhury et al., 2007) and its introgression from wild relative or other closely related species has been limited by cross-incompatibility and co-transfer of unwanted trait (Machuka, 2002; Singh et al., 2002; Popelka et al., 2004; Chaudhury et al., 2007). Thus, genetic transformation can help to address this problem so that any of the needed insect resistance traits can be introduced to the variety of interest (Machuka et al., 2002). This can be witnessed by the development of transgenic plants of different crops (for example cotton, maize, and pea) reported so far.

Even though genetic transformation has been used to develop transgenic crops with news traits, a number of pre-requisites have to be fulfilled. One of the pre-requisites is the availability of suitable regeneration procedure amenable to transformation protocol (Brar et al., 1997b; Popelka et al., 2006; Cardoza, 2008). In some crops, the protocols are well documented and the application of the protocols is a matter of capacity rather than availability. On the other hand, in some orphan crops likes cowpea, regeneration and transformation procedures are still challenging (Brar et al., 1997b; Somers et al., 2003; Chaudhury et al., 2007; Bakshi et al., 2011).

During the last few decades, cowpea has been subjected to *in vitro* studies ranging from simple regeneration to genetic transformation. Cowpea has been cultured *in vitro* using explants such as shoot tips, embryos (whole or sliced) from immature or mature seeds, cotyledons, cotyledonary node from germinated seedlings and leaf cuttings/discs. Regeneration was successfully obtained on a media supplemented with different plant growth hormones mainly BA (Table 8). However, there is no consistence in terms of the molar concentration used among the reports and the currently available regeneration protocols are optimized on a very few cultivars limiting the universal usage of the protocols. In some cases, these regeneration protocols were used for genetic transformation with low success rate. In addition to organogenesis, somatic embryogenesis was also used in cowpea *in vitro* regeneration by culturing explants on a medium supplemented with 2,4-D (2.3 - 4.52 µM) for embryo induction and 2,4-D (0.05-0.45 µM) and ABA (5 µM) for embryo maturation (Anand et al., 2000; Ramakrishnan et al., 2005). In both reports, the conversion rate of the induced somatic embryos into plantlets was low (5-32 %) which could limit the application of somatic embryogenesis in the genetic modification of cowpea.

Both *Agrobacterium*-mediated and direct transformations were used in cowpea genetic manipulations (Table 9). Of these methods, *Agrobacterium*-mediated transformation is the

most popular method. Using direct gene transfer methods, only five groups (four with biolistic and one with *in planta* electroporation) have reported the successful development of transgenic cowpea lines. According to the different reports (Table 9), transformants were achieved with better efficiency using *Agrobacterium*-mediated method (1.43-3.6 %) than the direct method (0.14-0.9 %). In most of the transformation reports, there is either one or two of the following problems: low transformation efficiency, no information on the inheritance of the transgene and/or lack of Mendelian inheritance of the introduced transgene. These problems underline the difficulty of producing transgenic lines in cowpea compounded by many factors ranging from the crop itself to the lack of robust protocols.

Cowpea is a very recalcitrant crop to *in vitro* regeneration and transformation (Brar et al., 1997b; Somers et al., 2003). Regeneration study on different cowpea varieties indicated that regeneration procedure has to be optimized for the variety of interest due to varietal difference (Brar et al., 1997b; Brar et al., 1999; Popelka et al., 2006). This has limited the use of the available regeneration and transformation protocols for the variety in hand. Moreover, cowpea is economically more important in resource limited areas of the world such as Africa which limited the application of modern technology such as genetic transformation to develop improved varieties (Machuka et al., 2002).

In general, there is no robust protocol for regeneration and transformation of cowpea posing challenge in the application genetic transformation in its improvement programs. Thus, fine-tuning of the existing regeneration protocols is needed in order to optimize protocol which could work across varieties.

The general aim of this part of the study was to develop transgenic cowpea with new agronomic traits through *Agrobacterium*-mediated transformation. In line with this aim, the following two objectives were pursued during the study time. The first objective was to optimize *in vitro* regeneration conditions suitable for *Agrobacterium*-mediated transformation of Kenya cowpea variety K80. The specific factors considered include (1) medium for (a) seed pre-conditioning, (b) multiple shoot induction, (c) root induction, (d) inoculation and (e) co-cultivation and (2) explant type. In the second objective, using the optimized protocol, attempts were made to develop insect resistant transgenic cowpea lines expressing either *cry1Ab* or *cry1Ac* gene.

Table 8. Summary of cowpea regeneration during the last few decades

Variety(cultivar)	Explant	Basic Medium	Hormone and concentration	Average number of shoots per explant	Reference
VITA 5-EXIITA	Shoot tip meristem	MSB_5	0-0.5 µM BA + 0-0.5 µM NAA	-	(Kartha et al., 1981)
Temine	Shoot tips	MS	0-0.2 µM IAA + 0-0.2 µM 2iP	-	(Sebastian, 1983)
C-152	Calli from primary leaves	B5	5 µM BAP	3.3	(Muthukumar et al., 1995)
C152	Calli from *in vitro* leaves	MS	0.5-2 mg/L 2,4-D	-	(Kulothungan et al., 1995)
Georgia-21	Shoot tips	MS	22.2 µM BA + 0.054 µM NAA or 0.045 µM 2,4-D	~5	(Brar et al., 1997a)
17 different genotypes	Cotyledons	MS	4.4 µM BA	4-12	(Brar et al., 1999)
EPACE-1	Longitudinal thin cell layers	MSB_5	10 µM TDZ for pre-treatment 1 µM IBA + 1 µM TDZ for bud proliferation	Up to 32.5 shoot buds	(Van Le et al., 2002)
Mognolia Blackeye	Immature embryo	B5	4.4 µM BA	10.1	(Choi et al., 2003)
C152	Calli from *in vitro* leaves	MSB_5, B5	0.1-0.5 mg/L 2,4-D and 0.05 mg/L TDZ	Somatic embryos followed by plantlets	(Ramakrishnan et al., 2005)
Blackeye	Shoot tips	MSB_5	8.88 µM BA	8	(Mao et al., 2006)
19 different cultivars	Embryonic axis from Immature seeds immature seeds	B5 or MS	7.51 µM BAP	 3.3-6.1 2.2-5.9	(Popelka et al., 2006)
V-585	CN from 4 days old seedling on MSB_5 + 10 µM BA	MSB_5	5 µM BAP	~6-6.3	(Chaudhury et al., 2007)
Mougne	CN with one cotyledon CN with two cotyledon CN without cotyledon	B5	4.4 µM BA	6.64 8.3 4.57	(Diallo et al., 2008)
IT86D1010	Decapitated embryo	MS	8.8 µM BA for induction, 2.2 µM BA for proliferation and 0.44 µM BA for Elongation	5-6	(Yusuf et al., 2008)
Four different cultivars	Shoot meristem from embryos precultured 3-5 days on MS medium containing 8.9 µM BA	MS	0.89 µM BA	6.29-6.89	(Manoharan et al., 2008)
IT86D1010	Decapitated embryo	MSB_5	2.2- 8.8 µM BA	?	(Raji et al., 2008)

Table 8. Continuation

Pusa Komal	CN	MSB_5	5 µM BA	6.9	(Solleti et al., 2008b)
Co(cp)-7	CN from MSB_5 + 13.3 µM BA	MSB_5	6.6 µM BA and then 0.5 µM BA	13.5	(Raveendar et al., 2009)
Akkiz	Shoot tips	MS	1.13 µM TDZ	4.72	(Aasim et al., 2009a)
Karagoz	Shoot tips	MS	1.13 µM TDZ	2.86	(Aasim et al., 2009a)
Akkiz	Plumular apices from embryo cultured on MS+ 44 µM BA for 10 days	MS	4.4 -5.5 µM BAP	6.42-7.11	(Aasim et al., 2009b)
Akkiz	DE from 5 days old embryo on MS + 44 µM BA	MS	4.4 µM BA + 0.54 µM NAA	10.33	(Aasim et al., 2010)
Rabo-de-tatu and Branco	Shoot tips	MS	1.1 µM BA	?	(do Rego et al., 2012)
Akkiz	Longitudinally sliced CN Unsliced CN	MS MS	3.33-4.44 µM BA 2.22 µM BA	9.79-9.92 9.33	(Aasim et al., 2012)
Cheng-jiange II	CN	MSB_5	5.3 µM BA	4.47	(Tang et al., 2012)
Eight cultivars	CN	MSB_5	5 µM BA + 0.5 µM Kinetin	1.14-6.72	(Bakshi et al., 2012a)
Akkiz	Immature cotyledon	MS	2.2 µM BA	5	(Aasim et al., 2013)
Cheng-jiange II	CN	MSB_5	2.2 -6.6 µM BA	4.59-5.53	(Tie et al., 2013)

Table 9. Three decades of cowpea transformation efforts

Date	Explants used	Transformation methods	Agrobacterium strain	Gene	Transgenic status	Remarks	Transformation efficiency	Reference
1986	Leaf disc from primary leaves	*Agrobacterium tumefaciens*	C58C1	Kanamycin resistant	Transgenic calli	No whole plant was regenerated	-	(Garcia et al., 1986)
1987	Leaf disc from primary leaves	*Agrobacterium tumefaciens*	C58C1	mRNA of cowpea mosaic virus(CPMV)	Transgenic calli	No transgenic plant was regenerated	-	(Garcia et al., 1987)
1991	Mature embryos	*Agrobacterium tumefaciens*	A281 and C58	*uidA*	Transgenic calli and Chimeric transformants	No transgenic plant was regenerated	-	(Penza et al., 1991)
1992	Mature embryos	Biolistic	-	*uidA*	Transient expression		-	(Penza et al., 1992)
1993	Mature embryos	Biolistic	-	*uidA*	Transient expression		-	(Akella and Lurquin, 1993)
1995	Nodal meristems	*In planta* electroporation	-	*uidA*	Transgenic plants			(Chowrira et al., 1995; Chowrira et al., 1996)
1996	De-embryonated cotyledon from 2-3 day old seedling	*Agrobacterium tumefaciens*	LBA4301	*Hygromycin phosphotransferase (hpt)*	Hygromycin resistant Primary transformants	Progeny from primary transformant was not reported	Not indicated	(Muthukumar et al., 1996)
1997	Embryo from immature seeds	*Agrobacterium tumefaciens* Biolistic	LB4404	*nptII, αAI-1*, *uidA* and *bar*	Primary transformants	Inheritance of the transgene was not reported.	0.75 % 0.2 %	(Kononowicz et al., 1997)
2003	Mature embryos	Biolistic	-	*uidA* and *bar*	Transgenic plants	Transgene was not inherited in a Mendelian laws	0.14 %	(Ikea et al., 2003)
2006	Decapitated embryo attached cotyledon	*Agrobacterium tumefaciens*	AGL1	*uidA* and *bar*	Transgenic plants	Transgene inheritance in a Mendelian laws was confirmed in progeny	0.05-0.15 %	(Popelka et al., 2006)
2007	CN from 4 day old seedling	*Agrobacterium tumefaciens*	EHA105	*uidA* and *nptII*	Transgenic plants	Transgenic progeny	0.76 %	(Chaudhury et al., 2007)
2008	Decapitated embryo axes	*Agrobacterium tumefaciens*	LBA4404	*uidA* and *npt*	Primary transformant	No information on progeny analysis	2.96 %	(Raji et al., 2008)
2008	Embryonic axes	Biolistic	-	*ahas* and *uidA*	Transgenic progeny	T2 generation	0.90 %	(Ivo et al., 2008)

Table 9. Continuation

Date	Explants used	Transformation methods	Agrobacterium strain	Gene	Transgenic status	Remarks	Transformation efficiency	Reference
2008	Nodal buds	*In planta* electroporation	-	*Cry1Ab* and *NptII*	Transgenic plants	The transgene inheritance did not follow Mendelian laws	Not indicated	(Adesoye et al., 2008)
2008	CN from three day old seedling	*Agrobacterium tumefaciens*	LBA4404	*NptII* and *uidA*	Transgenic lines expressing nptII and *gus* genes	T1 generation with Mendelian inheritance of the transgene	1.64 %	(Solleti et al., 2008b)
2008	CN from three day old seedling	*Agrobacterium tumefaciens*	LBA4404	*NptII*, *αAI-1* and *uidA*	Transgenic progeny	Mendelian inheritance of αAI-1 in T1 progeny was confirmed.	1.67 %	(Solleti et al., 2008a)
2010	CN	*Agrobacterium tumefaciens*	LBA4404	*uidA* and *hpt*	Primary transformant	Inheritance to progeny was not reported	1.61 %	(Raveendar and Ignacimuthu, 2010)
2010	Embryo	Vacuum assisted *Agrobacterium tumefaciens*	pGV3850 pGV2260	*Bar* and *uidA* *hpt* and *uidA*	Transgenic progeny	Data on the Mendelian inheritance of the transgene was not provided	2.5 % 3.9 %	(Adesoye et al., 2010)
2011	CN from three day old seedling	*Agrobacterium tumefaciens*	EHA105	*NptII*, *cry1Ac* and *uidA*	Transgenic plants	Mendelian inheritance of cry1Ac gene in T1 progeny was indicated	3.09 %	(Bakshi et al., 2011)
2012	CN	*Agrobacterium tumefaciens*	EHA105	*NptII* and *uidA*	Transgenic progeny	Mendelian inheritance of nptII gene in T1 progeny was indicated	0.6-2.1 %	(Bakshi et al., 2012a)
2012	CN from 4 day old seedling	*Agrobacterium tumefaciens*	EHA105	*Phosphomannose isomerase(pmi)*	Transgenic progeny	Mendelian inheritance of *pmi* gene in T1 progeny was indicated	3.6 %	(Bakshi et al., 2012b)
2013	Immature cotyledon	*Agrobacterium tumefaciens*	LBA4404	*bar* and *uidA*	Putative transgenic plants	Mendelian inheritance of the transgene was not provided in the progeny	1.5 %	(Aasim et al., 2013)
2013	Embryonic axes from mature seeds	Biolistic	-	*atahas and uidA*	Imazapyr-tolerant lines	Mendelian inheritance of transgene at T1 generation was indicated	Not indicated	(Citadin et al., 2013)

Adapted and updated from different sources (Citadin et al., 2011; Diouf, 2011; Manman et al., 2013)

4.3 Materials and methods

4.3.1 Plant material

Seed of the Kenyan cowpea variety K80 (Fig. 17) were used for optimization of regeneration and transformation conditions as well as for stable transformation with *B.t cry* genes.

Fig. 17 Seeds of Kenyan cowpea variety K80 collected from greenhouse grown plants.

4.3.2 *Agrobacterium* strains and transformation vectors

4.3.2.1 Transient transformation

For transient transformation, a transformation vector harboring *GUS* (pIBGUS) and *GFP* (pEGAD-GFP) genes were used. The pIBGUS vector (de Kathen and Jacobsen, 1990) in *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) was used for transient GUS expression. Fig. 18 shows the physical map of the pIBGUS vector T-DNA region. The transformation vector contains a *35S*-promoter driven *GUSA*-gene with an ST-LS1 intron reporter gene as well as a *35S*-promoter driven *pat* gene and a *Nos-NPTII* gene as plant selectable markers (de Kathen and Jacobsen, 1990; Krishnamurthy et al., 2000).

Fig. 18 The physical map of the pIBGUS transformation vector T-DNA region (Dr. Fathi Hassan, personal communication). The arrows show the direction of transcription.

The pEGAD-GFP vector (Cutler et al., 2000) in *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) was used for transient GFP expression. Fig. 19 shows the physical map of the pEGAD-GFP vector T-DNA region. The *GFP* gene is under the control of *35S* promoter from CaMV and *OCS* terminator from *Agrobacterium* and the *bar* gene is under the control of *35S* promoter and terminator from CaMV.

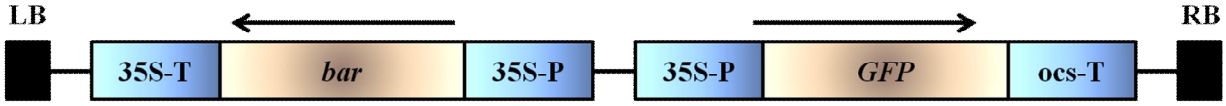


Fig. 19 The physical map of the pEGAD-GFP transformation vector T-DNA region (Dr. Fathi Hassan, personal communication). The arrows show the direction of transcription.

4.3.2.2 Stable transformation

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) carrying a dual binary vector pGreenII/pSoup, pGIIMH35s (Hellens et al., 2000) was used for this purpose. The binary vector harbors the gene of interest (either *cry1Ac* or *cry1Ab)* and the *bar* gene between its right and left borders. Fig. 20 shows the functional map of the transformation vector for both genes.

The *cry* genes (*cry1Ac* and *cry1Ab*, 1.845 kb each) are under the control of double *35S* promoter from CaMV and nopaline synthase (*nos)* terminator from *Agrobacterium tumefaciens*. They are plant codon usage optimized insect resistance genes from *Bacillus thuringiensis* (Sardana et al., 1996; Cheng et al., 1998). They encode crystal proteins toxic to insect pests in the Lepidopteran order (Hofte and Whiteley, 1989).

The *bar* gene from *Streptomyces hygroscopicus* (Murakami et al., 1986; Thompson et al., 1987) under the control of a *nos* promoter and terminator sequence of *Agrobacterium tumefaciens* is used as a plant selectable marker gene. It encodes the enzyme phosphinothricin acetyltransferase (PAT) and confers resistance to bialaphos, glufosinate

ammonium and phosphinothricin (PPT), the active compounds of the total herbicide BASTA®, through acetylation (Thompson et al., 1987; Lindsey, 1992; Finer and Dhillon, 2008; Miki, 2008).

The vectors also contain an *NptI* gene outside the T-DNA region for Kanamycin resistance. The *NptI* gene enables selective mass production of the bacterial cells in/on Kanamycin containing medium.

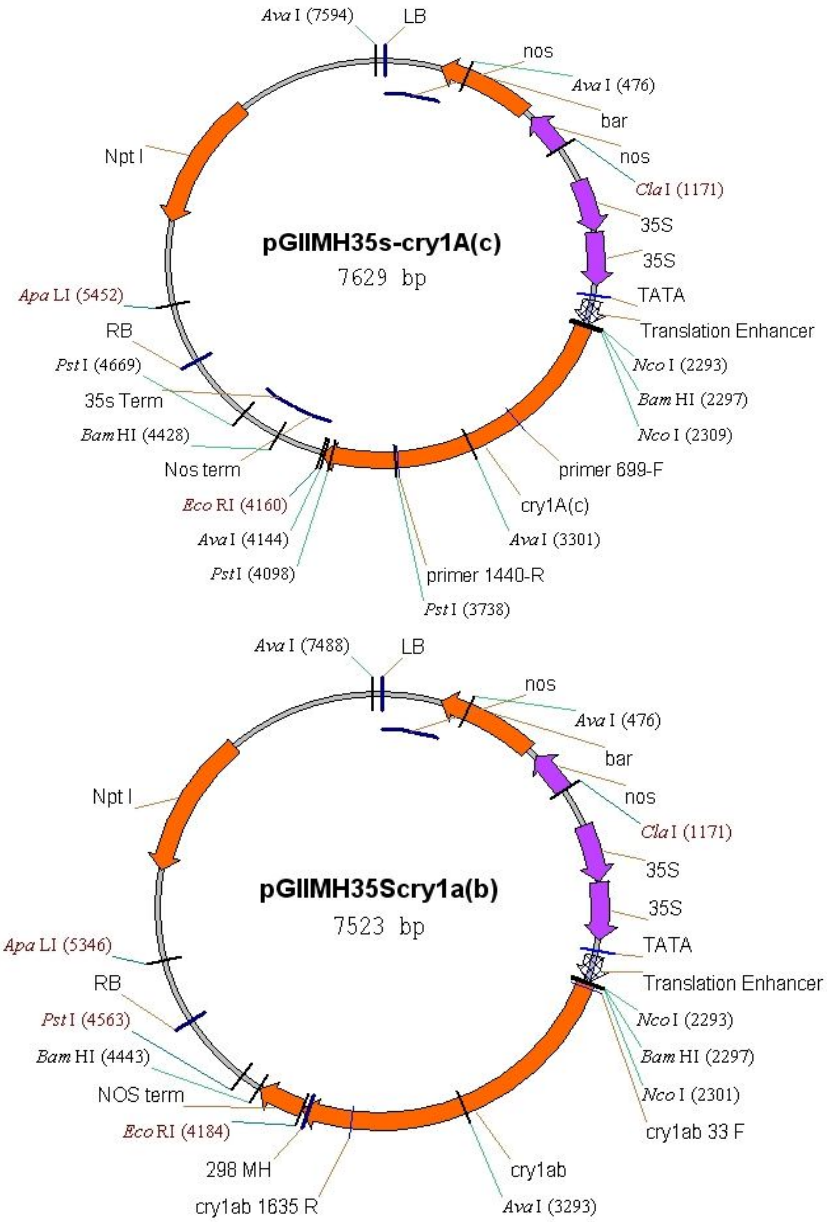


Fig. 20 The functional map of the transformation vector harboring *cry1Ac* or *cry1Ab* gene (Kefale, 2006).

4.3.3 Seed disinfection and explant preparation

For explant preparation, cowpea seeds were surface sterilized using the procedure for pea seeds with little modification as follows: first the seeds were washed with tap water and treated with 70 % (v/v) ethanol for one minute followed by 3-5 times rinsing with sterile distilled water. Then the seeds were treated under the clean bench with 6 % sodium hypochlorite (with a drop of Tween20) for 30 minutes with occasional shaking (preferably on a shaker at ~120 rpm). The treated seeds were thoroughly washed with sterile distilled water (4–5 times) to remove any trace of sodium hypochlorite. The surface sterilized seeds were imbibed overnight (~12 hr) in sterile distilled water (preferably on a shaker at ~120 rpm and 26-28°C). The overnight imbibed seeds were used to prepare two types of explant: decapitated embryo (DE) and cotyledonary node (CN).

For DE preparation (Fig. 21a), the seeds were carefully cut and opened. Then, the seed coat and cotyledons were removed, and the DE was obtained by removing the root and shoot tips of the embryos.

For CN preparation (Fig. 21b), the surface sterilized and overnight imbibed seeds were germinated on a pre-conditioning medium for 3-4 days at 22 ± 2 °C and 16 hr photoperiod. Then, the CN was isolated and used for the experiment.

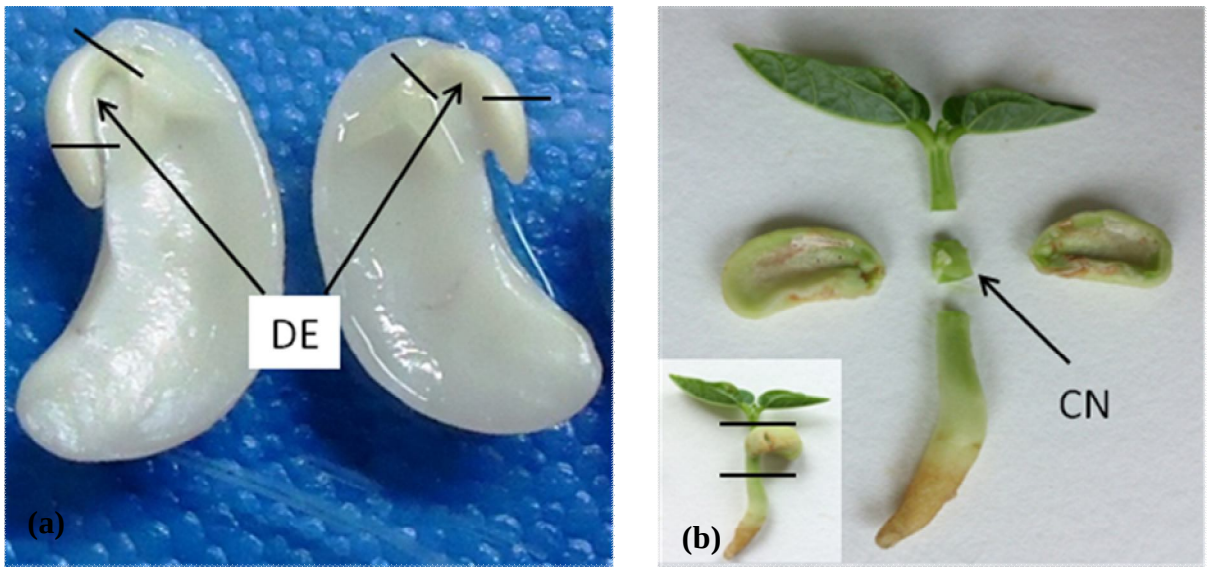


Fig. 21 Explants preparation for *in vitro* regeneration and transformation of cowpea: a) Decapitated embryo and b) Cotyledonary node.

4.3.4 Optimization of the *in vitro* conditions for regeneration and *Agrobacterium*-mediated transformation of cowpea

For cowpea transformation, there is no universally applicable and genotype neutral protocol yet. Hence, the existing protocols need to be optimized for the specific variety of interest. Because of this gap, different *in vitro* conditions were optimized in a series of experiments. The optimized conditions include media (for shoot regeneration, pre-conditioning, inoculation/co-cultivation and selective regeneration), explant type and *Agrobacterium* concentration. Then, based on the optimized protocol, stable transformation experiment with *cry1Ac* and *cry1Ab* genes were attempted to develop insect resistant cowpea lines. The detailed description of the protocol optimization and transformation experiments is given in the following sub-sections.

4.3.4.1 Effect of BA alone or in combination with either Kin or NAA on multiple shoot production in cowpea using cotyledonary node explants

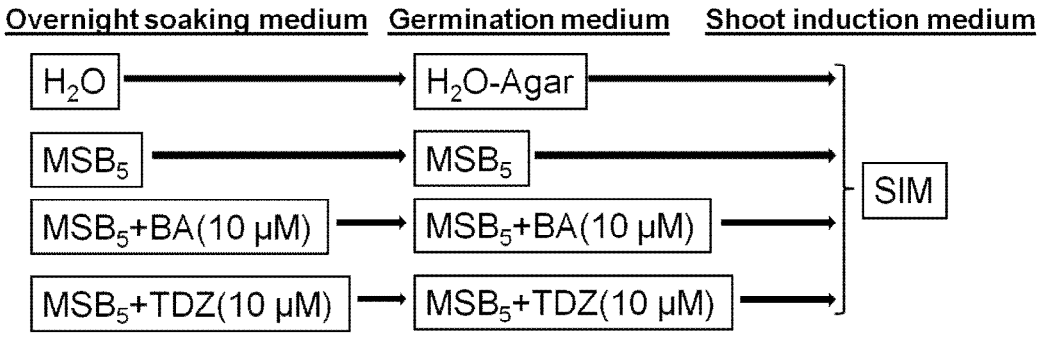
Ten BA concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µM) were tested alone or in combination with 0.5 µM Kinetin or 0.5 µM NAA. The experiment was conducted in completely randomized design with three replications. Twelve cotyledonary node explants per treatment per replication were used. The performance of the explants were evaluated (Fig. 22) after three weeks for the regeneration efficiency (0-1), presence of callus (scale: 0,1,2,3,4, and 5 with 0 means no callus and 5 means big callus size), number of shoots, the length of shoot (cm), the number of root and the length of roots (cm). The culture room conditions were 22 ± 2 °C and 16/8 hr photoperiod.

Fig. 22 Evaluation parameters of cowpea regeneration from CN explants

4.3.4.2 Effect of pre-conditioned medium on multiple shoot production from CN explants

The most commonly used explants in cowpea regeneration are cotyledonary nodes from 3-4 days old seedlings germinated on pre-conditioning media. The effect of seed soaking and germination media on multiple shoot induction was studied. The media included Water-Agar, MSB_5, MSB_5 + 10 µM BA and MSB_5 + 10 µM TDZ. In addition, in a separate experiment, the effect of different levels of either BA or TDZ (0-15 µM) were also tested as pre-conditioning medium supplements. After surface sterilization, seeds were treated as indicted in Fig. 23a. Fig. 23b shows seedling germinated on different pre-conditioning media. The cotyledonary nodes obtained from each treatment were placed on the same shoot induction medium. After three weeks, the number shoots per explant was recorded and then subjected to further analysis.

(a)



(b)

Fig. 23 Seed pretreatment: (a) overnight soaking and pre-conditioning schemes for seeds and (b) seedlings germinated on the different pre-conditioning media and used for preparation of CN explants.

4.3.4.3 Effect of inoculation and co-cultivation medium on multiple shoot production of cowpea explants

Beside overnight soaking and pre-conditioning media, the effect of explant type (DE and CN) and inoculation/co-cultivation media were also studied on multiple shoot induction. Fig. 24 shows the scheme of explants inoculation and co-cultivation during *Agrobacterium*-mediated transformation. Following this scheme, explants were treated with the different media (without *Agrobacterium*) and then evaluated for multiple shoot production on shoot induction medium.

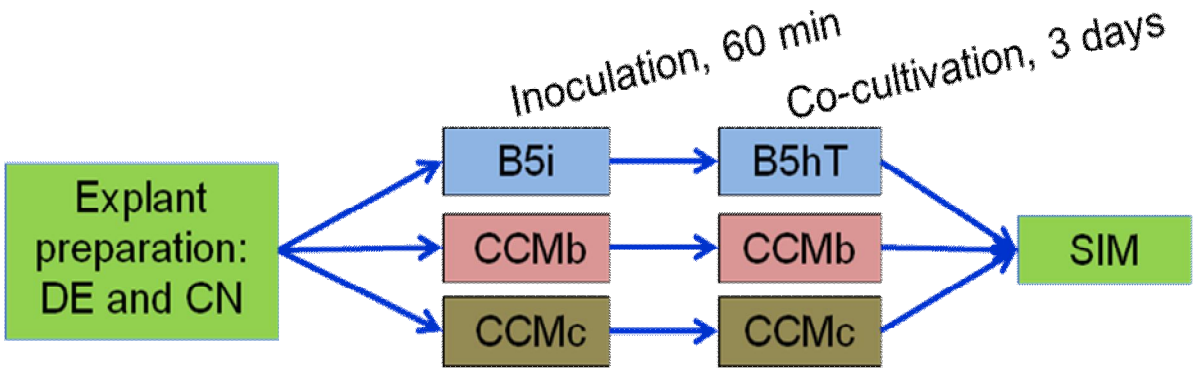


Fig. 24 Schematic representation of explants treatment with inoculation and co-cultivation medium and then culturing on shoot induction medium (SIM). B5i, B5hT, CCMb and CCMc are media used during inoculation and co-cultivation.

4.3.4.4 Effect of IBA on rooting characteristic of cowpea *in vitro* shoots

For *in vitro* rooting, actively growing shoots were cultured on MSB_5 medium supplemented with different levels of IBA (0, 1, 2, 2.5, 3, 4, 5, 10, 15 and 20 µM). The rooting characteristic of the shoots were evaluated for the presence of roots, the number of roots and the length of roots (cm) (Fig. 25).

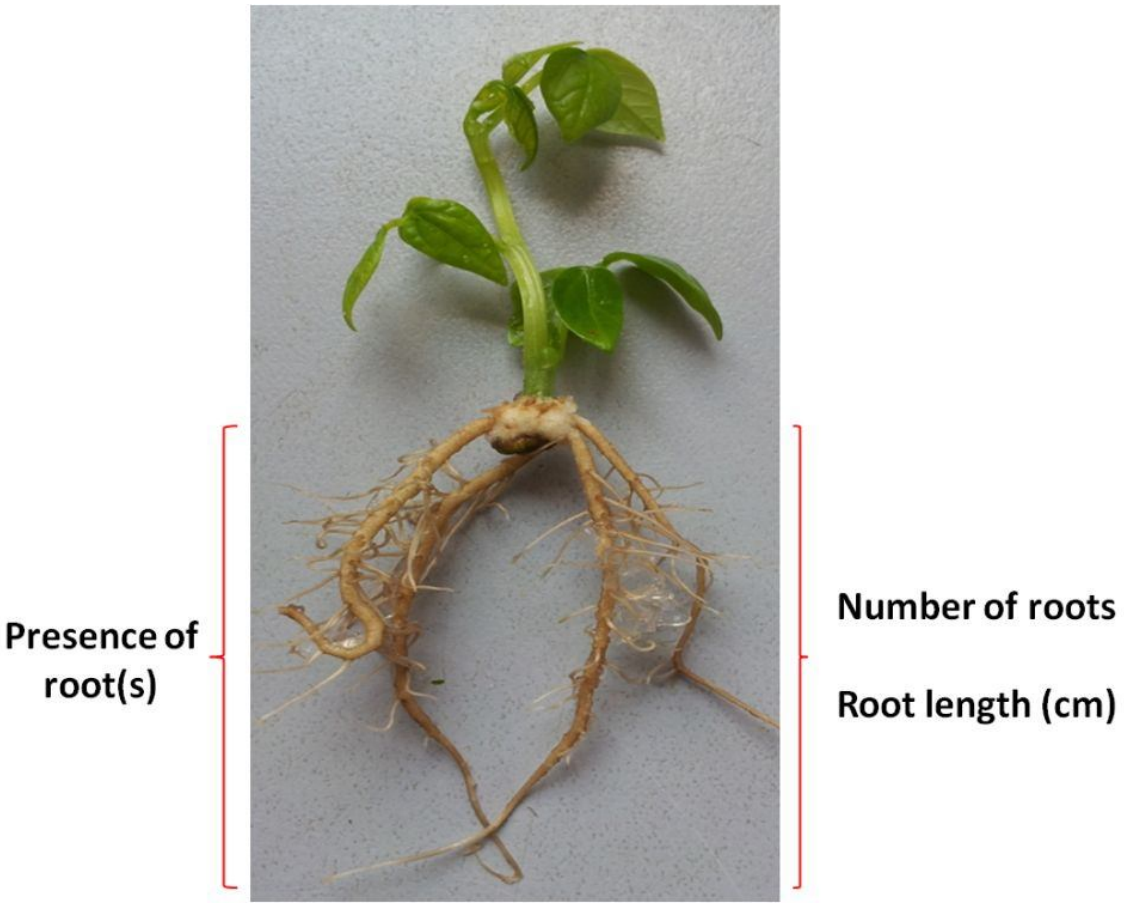


Fig. 25 Evaluation of *in vitro* rooting of cowpea shoots

4.3.4.5 Optimization of phosphinothricin (PPT) concentration for selective regeneration of putative transgenic shoots and functional characterization of cowpea plants

To test the sensitivity of cowpea to PPT and Basta® herbicide solution, two experiments were conducted. In the first experiment, the sensitivity of *in vitro* shoots was tested on a medium supplemented with PPT. Both primary shoots from *in vitro* germinated seeds on water-agar medium and secondary shoots from cotyledonary nodes cultured on shoot induction medium (MSB_5 + 3 µM BA +0.5 µM Kin) were used for this experiment. The shoots were cultured on shoot induction medium supplemented with different concentration of PPT (1-4.5 mg/L). The experiment was laid in a completely randomized design with three replications. Twelve explants were used per treatment per replication. The culture room conditions were 22 ± 2 °C

and 16/8 hr photoperiod. The percentage of surviving shoots was recorded after three weeks and the data were subjected to analysis.

In the second experiment, leaf paint functional assay (Schroeder et al., 1993) was used to determine the sensitivity of cowpea plants to BASTA® (200g/l stock) herbicide solutions. Seeds of cowpea (variety K80) were grown in the green house and used for the assay. The herbicide solutions (with a drop of Tween20) at different levels of the active ingredient (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg/L) were prepared and applied onto the upper side of the leaves. The sensitivity of the leaves was evaluated visually one week after application.

4.3.4.6 Optimization of inoculation and co-cultivation conditions using transient transformation

Transient transformation was conducted to optimize inoculation and co-cultivation conditions. Transformation vectors harboring *GUS* (EHA101 pIBGUS) and *GFP* (EHA105 pEGAD-GFP) genes were used. Glycerol stock of *Agrobacterium* was grown overnight in YEP medium supplemented with 50 mg/L Kanamycin on a shaker at 28°C. The overnight culture was harvested by centrifugation (4,500 rpm and 4 °C) and used for transformation of explants after re-suspended in the inoculation medium.

1.1.1.1.1 Effect of inoculation and co-cultivation media on transient transformation of DE and CN explants

To test media for inoculation and co-cultivation, three paths of transformation were followed (Fig. 26). The first path was based on the procedure for pea transformation (Schroeder et al., 1993) using B5i medium (B5 medium + 10 g/L sucrose + 10 g/L glucose + 2 g/L MES + 100 µM acetosyringone, pH 5.6) for inoculation and B5hT medium (B5 medium + 1 µM Kinetin + 5 µM TDZ + 7.4 µM Adenine + 0.88 g/L $CaCl_2.2H_2O$ + 0.5 g/L KNO3 + 0.5 g/L MgSO4.7H2O + 0.8 g/L Glutamine + 10 mg/L Glutathione + 30 g/L sucrose + 2 g/L MES + 4.5 g/L Gelrite, pH 5.5) for co-cultivation. The second path was based on the protocol reported by Solleti et al. (2008a) using MSB_5 medium (pH 5.5) supplemented with 1µM BAP, 1mM dithiothreitol and 8.3 mM L-Cysteine and 100 µM acetosyringone for inoculation (liquid medium) and co-cultivation (semi-solid medium, 7.5 g/L plant agar)[CCMb]. The last

path was similar to the second scheme except 3 µM BAP and 0.5 µM Kinetin were used instead of 1 µM BAP [CCMc: MSB_5 medium pH 5.5 supplemented with 3 µM BAP, 0.5 µM Kinetin, 1mM dithiothreitol and 8.3 mM L-Cysteine and 100 µM acetosyringone]. In all the transformation schemes 60 min inoculation time and three days of co-cultivation were used. GUS expression was detected as described by Jefferson et al. (1987) and the expression of GFP was analyzed under the microscope using UV lamp with filter.

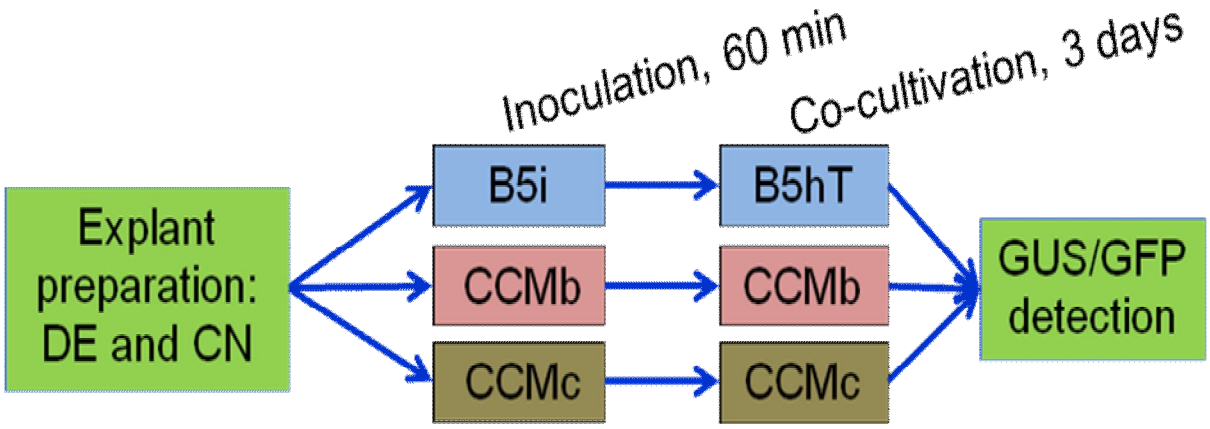


Fig. 26 Inoculation and co-cultivation schemes for transient transformation of cowpea.

1.1.1.1.2 Sonication and vacuum infiltration assisted transient transformation in cowpea

Sonication and vacuum infiltration assisted *Agrobacterium*-mediated transformation was also conducted to further improve transformation efficiency using the CCMb medium for inoculation and co-cultivation. The transformation vector containing the *GUS* gene was used for this experiment. Three levels of sonication intensity (0, 30 and 60 seconds) and three levels of vacuum infiltration (0, 2.5 and 5 minutes) were applied on the explants. As explants embryos from overnight soaked seeds were used.

1.1.1.1.3 Effect of bacterial culture concentration on transient transformation

The effect of bacterial concentration on the transient transformation was tested using the *GUS* construct and DE explants. An overnight culture of *Agrobacterium* (OD_{600}=1-1.5) was harvested (at 4,500 rpm and 4°C) and re-suspended in inoculation medium [CCMbT= modified CCMb medium containing higher concentration of acetosyringone (200 µM) and 1 mM Na-thiosulphate]. The OD_{600} of the re-suspended *Agrobacterium* was adjusted to 0.5,

1.0, 1.5 and 2.0 and then used for transformation of DE explants. The transformed explants were co-cultivated on CCMbT medium in the dark at 22±2 °C for three days. GUS assay was conducted as described by Jefferson et al. (1987). Transformation efficiencies (TE, %) and the intensity of GUS expression were determined at the different levels of bacterial concentration.

4.3.4.7 *Agrobacterium*-mediated transformation of cowpea with *B.t cry* genes

Based on the optimized conditions, transformation experiments were conducted with transformation vectors harboring the *cry1Ac* or *cry1Ab* gene for insect resistance. In the regenerated putative transgenic shoots, genomic integration of the transgene was analyzed by PCR using transgene specific primers.

4.4 Results

4.4.1 Regeneration of cowpea from cotyledonary node explants

Kenyan cowpea variety (K80) was regenerated on MSB_5 medium containing BA alone or in combination with Kinetin or NAA. Regeneration performance of explants was evaluated for regeneration frequency (shoot formation), callus induction, shoot number and length, as well as root number and length. Except regeneration efficiency, other parameters were significantly affected on the tested media (Table 10).

The regeneration efficiency ranged from 0.87-1.00 and there was no significant difference among the different media. Callus was observed on all media containing plant growth hormones and was significantly affected on media containing different molar concentration of plant hormones. The biggest callus size was recorded on media containing higher molar concentrations of BA (6-10 µM). The size of the induced callus increased with the increase in the molar concentration of BA in the medium.

The addition of BA alone or in combination with kinetin or NAA to the medium had a significant effect on the number of shoots per CN explants. The average number of shoots per explant ranged from 1.4 to 4.8. The highest number of shoots (4.8) per explant was observed on medium containing 3 µM BA and 0.5 µM Kinetin. Statistically the same average number

of shoots per explant was obtained on a medium containing 5 µM BA. On the other hand, the number of shoots per explant obtained on media containing 2-4 µM BA alone or in combination with kinetin or NAA and 6-7 µM BA was not significantly different from the number of shoots per explant obtained on medium containing 5 µM BA. The lowest number of shoots per explants was observed on a medium with low or no growth hormone or on a medium containing high concentration of BA.

The observed shoot length ranged from 2.16-8.82 cm and was significantly affected on media supplemented with different molar concentrations of BA. The highest shoot length (8.82 cm) was obtained on a medium with no plant hormones. On a medium containing 1 µM BA, shoot length was reduced to 4.07 cm and the shortest shoot length was obtained on medium containing 5 µM BA and 0.5 µM NAA. Statistically the same shoot length was obtained on medium supplemented with 1-3 µM BA alone or in combination 0.5 µM Kinetin or NAA. Shoot length became much shorter (2.16-3.80 cm) when moderate to high concentration of BA was used in the media.

On the regeneration medium, the explants were also evaluated for the rooting characteristics. Accordingly, the result showed that the addition of growth hormones into the regeneration medium had a significant effect on the rooting characteristics of the explants. On plant growth hormone free medium, the average root number and length (cm) per explants was 4.56 and 6.33, respectively, and both root number (1.56 per explant) and length (0.71 cm) were significantly reduced on a medium supplemented with 1 µM BA. On media containing 2-10 µM BA alone or in combination with kinetin or NAA, almost all the explants did not produce roots.

Table 10. The regeneration performance on cowpea var. K80 using cotyledonary node explants from three days old germinated seedlings

Medium code	MSB_5 medium plus			R*	C*	SN*	SL*	RN*	RL*
	BA(µM)	Kinetin (µM)	NAA(µM)						
C0	0	0	0	0.96 [a]	0.00 [h]	1.52 [de]	8.82 [a]	4.56 [a]	6.33 [a]
C1	1	0	0	0.88 [a]	1.28 [g]	1.40 [e]	4.07 [bcd]	1.56 [b]	0.71 [b]
C1K	1	0.5	0	0.87 [a]	1.67 [fg]	2.60 [bcde]	2.95 [bcde]	0.00 [c]	0.00 [b]
C1N	1	0	0.5	0.94 [a]	2.29 [cdefg]	2.09 [cde]	4.40 [b]	0.12 [c]	0.10 [b]
C2	2	0	0	1.00 [a]	2.27 [defg]	3.24 [bc]	4.24 [bc]	0.00 [c]	0.00 [b]
C2K	2	0.5	0	1.00 [a]	3.78 [ab]	3.30 [bc]	3.35 [bcde]	0.00 [c]	0.00 [b]
C2N	2	0	0.5	0.94 [a]	2.31 [cdefg]	2.83 [bcde]	3.58 [bcde]	0.03 [c]	0.01 [b]
C3	3	0	0	1.00 [a]	2.32 [cdefg]	2.88 [bcde]	3.80 [bcde]	0.00 [c]	0.00 [b]
C3K	3	0.5	0	1.00 [a]	3.40 [abcd]	4.80 [a]	3.68 [bcde]	0.00 [c]	0.00 [b]
C3N	3	0	0.5	1.00 [a]	2.94 [bcdef]	3.15 [bc]	3.29 [bcde]	0.06 [c]	0.01 [b]
C4	4	0	0	0.96 [a]	2.56 [bcdef]	2.80 [bcde]	2.51 [cde]	0.00 [c]	0.00 [b]
C4K	4	0.5	0	0.90 [a]	1.10 [g]	2.40 [bcde]	2.41 [de]	0.00 [c]	0.00 [b]
C4N	4	0	0.5	0.91 [a]	3.18 [abcde]	2.91 [bcde]	2.45 [de]	0.00 [c]	0.00 [b]
C5	5	0	0	1.00 [a]	2.06 [efg]	3.64 [ab]	3.36 [bcde]	0.00 [c]	0.00 [b]
C5K	5	0.5	0	0.87 [a]	3.00 [abcde]	2.20 [bcde]	2.20 [e]	0.00 [c]	0.00 [b]
C5N	5	0	0.5	0.94 [a]	3.09 [abcde]	2.66 [bcde]	2.16 [e]	0.00 [c]	0.00 [b]
C6	6	0	0	0.90 [a]	3.10 [abcde]	3.00 [bcd]	2.87 [bcde]	0.00 [c]	0.00 [b]
C7	7	0	0	0.96 [a]	3.38 [abcd]	2.31 [bcde]	2.77 [bcde]	0.00 [c]	0.00 [b]
C8	8	0	0	1.00 [a]	3.25 [abcde]	2.29 [bcde]	2.68 [bcde]	0.00 [c]	0.00 [b]
C9	9	0	0	0.95 [a]	4.05 [a]	1.81 [cde]	2.56 [cde]	0.05 [c]	0.08 [b]
C10	10	0	0	0.95 [a]	3.59 [abc]	2.14 [bcde]	2.34 [e]	0.00 [c]	0.00 [b]

*Means followed by the same letter within column are not significantly different (REGWQ, p=0.05); R: Regeneration efficiency (0-1), C: Callus size (0-5), SN: Shoot Number, SL: Shoot Length (cm), RN: Root Number and RL: Root Length (cm).

4.4.2 Effect of pre-conditioning medium on multiple shoot production from CN explants

Two experiments were conducted to study the effect of pre-conditioning media on multiple shoot production. In the first experiment, CN from seedling pre-conditioned on water-Agar medium, MSB_5 medium and MSB_5 medium supplemented with either 10 µM BA or TDZ were cultured on the multiple shoot induction medium. The result indicated that CN from pre-conditioning medium supplemented with 10 µM TDZ produced significantly less number of shoots per explants (Fig. 27). There was no statistically different numbers of shoots per explant from CN obtained from the other three pre-conditioning media (water-Agar, MSB_5 and MSB_5 with 10 µM BA).

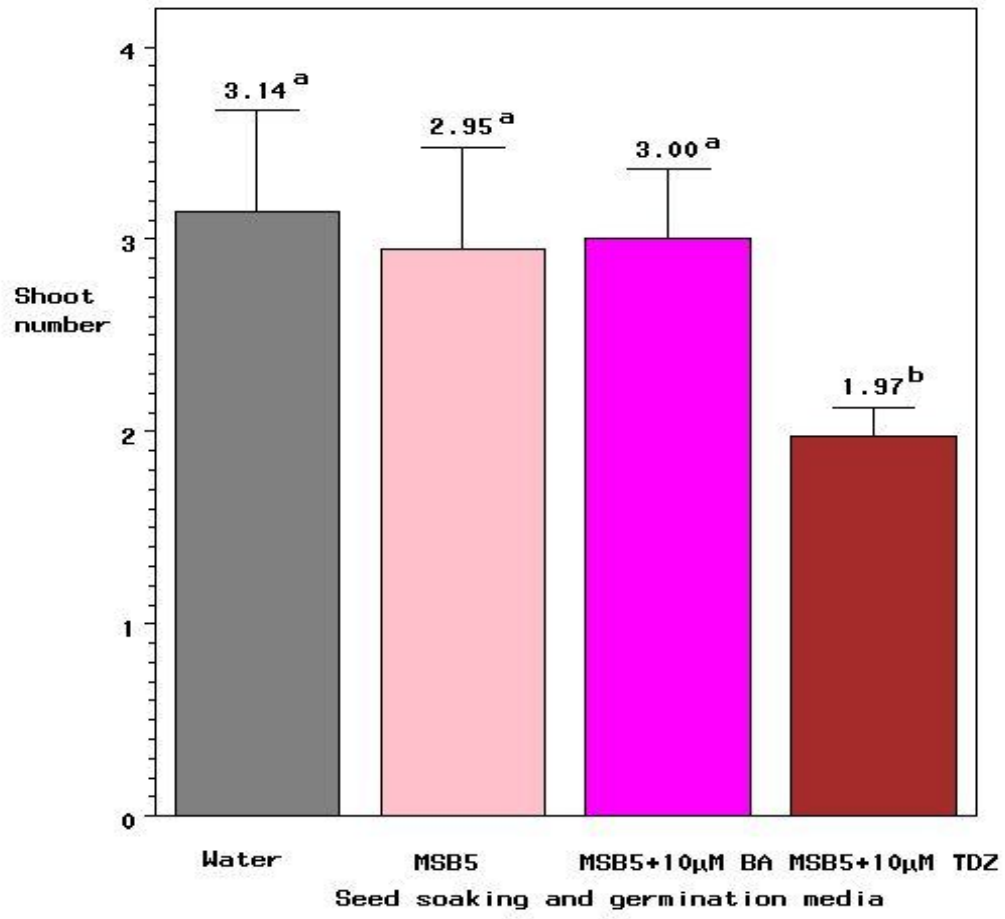


Fig. 27 Effect of seed soaking and germination media (pre-conditioning) on multiple shoot induction from CN explants. Means followed by the same letter are not significantly different (REGWQ, p=0.05).

In the second experiment, regeneration from CN explants obtained from seedlings germinated on a medium containing different concentrations of BA or TDZ were studied for multiple shoot induction. The result showed the number of shoots per CN explant was significantly affected by pre-conditioning media, while shoot length was not (Table 11). CN explants obtained from media containing 2.5-15 µM TDZ produced significantly less numbers of shoots per explant compared to those obtained from media supplemented with similar concentrations of BA or water-agar medium. The number of shoots per explant did not show difference when media with or without BA were used for seed germination.

Table 11. Effect of BA and TDZ supplement in pre-conditioning media on multiple shoot induction in cowpea

Plant growth regulator	Concentration (µM)	Shoot number*	Shoot length*
Control	-	2.74^{abcd}	3.48^{a}
BA	1	2.90^{abc}	3.47^{a}
	2.5	3.13^{ab}	3.36^{a}
	5	2.60^{bcd}	3.85^{a}
	10	3.33^{a}	3.38^{a}
	15	3.17^{ab}	3.20^{a}
TDZ	1	2.75^{abcd}	3.27^{a}
	2.5	2.30^{cd}	3.26^{a}
	5	2.43^{cd}	3.67^{a}
	10	2.39^{cd}	3.53^{a}
	15	2.18^{d}	3.24^{a}

*Means followed by the same letter within a column are not significantly different (REGWQ, p=0.05).

4.4.3 Effect of inoculation and co-cultivation media and explant type on cowpea *in vitro* regeneration

For transformation, explants are inoculated and co-cultivated with *Agrobacterium* in/on suitable media, i.e.; on a medium that supports both the growth of explants and infection with *Agrobacterium*. In this section, the effect of three inoculation/co-cultivation media on regeneration efficiency and multiple shoot induction from CN and DE explants were studied.

The use of different inoculation and co-cultivation media had significant effects on the regeneration efficiency of cowpea from CN explants (Fig. 28). However, regeneration

efficiencies (ranged from 0.86 to 0.96) from DE explants were not affected by the inoculation and co-cultivation media. For CN explants, the highest regeneration efficiency (0.970) was obtained when the explants were placed directly on shoot regeneration medium without pretreatment followed by explants treated with CCMb medium as inoculation and co-cultivation medium (0.949). Next to CCMb, CN explants treated with CCMc medium as inoculation and co-cultivation medium showed better regeneration efficiency (0.75), while the poorest regeneration efficiency was obtained when B5i/B5hT media were used for inoculation/co-cultivation.

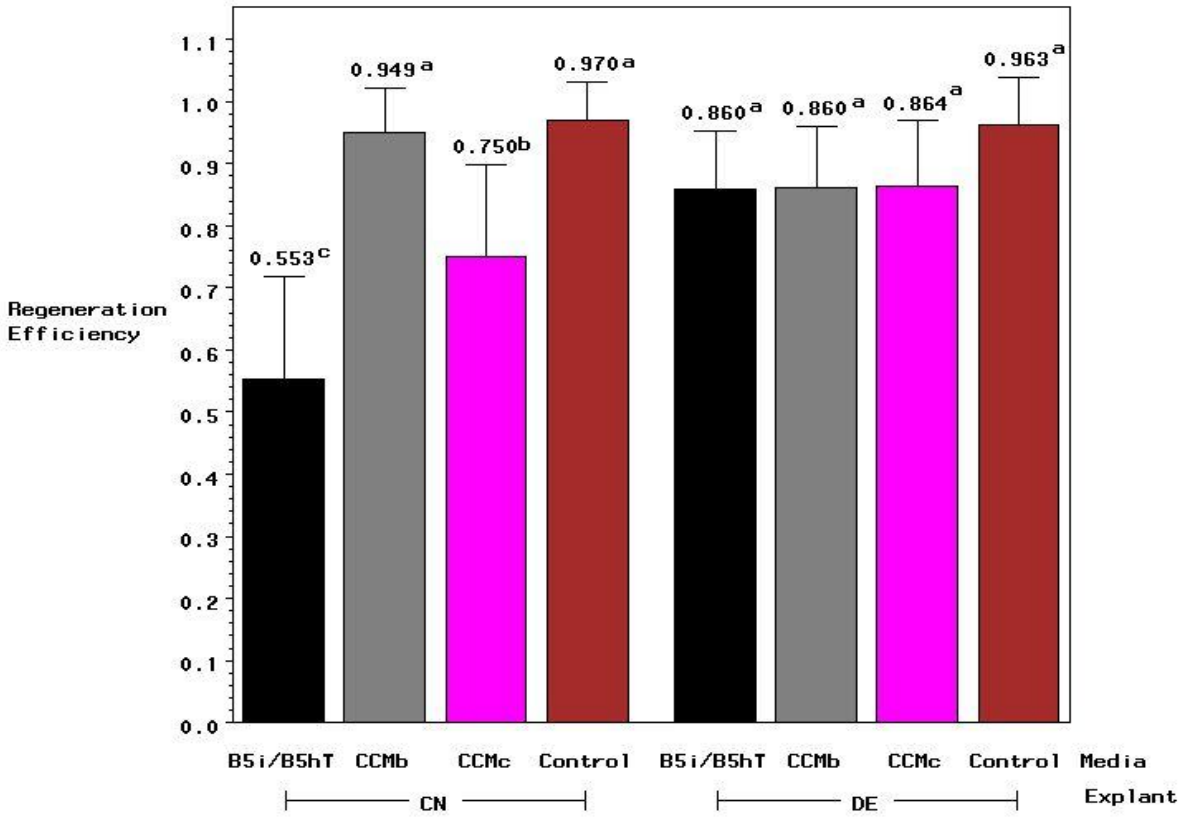


Fig. 28 Effect of inoculation and co-cultivation media on regeneration efficiency of cowpea explants. Means followed by the same letter within a group (explant) are not significantly different (REGWQ, p=0.05).

In contrast to the regeneration efficiencies, shoot number per CN and DE explants was not affected by the use of different media for inoculation and co-cultivation (Fig. 29). The shoot number per explant ranged from 2.43 to 3.34 for CN explants while it ranged from 1.78 to 2.37 for DE explants.

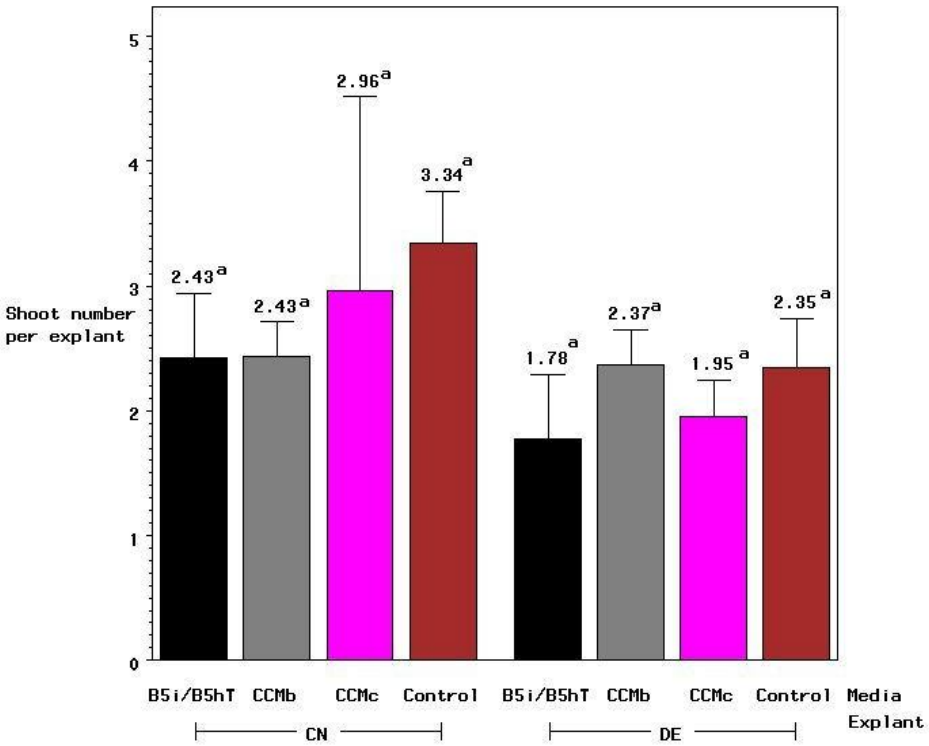


Fig. 29 Effect of inoculation and co-cultivation media on multiple shoot production from cowpea explants. Means followed by the same letter within a group (explant) are not significantly different (REGWQ, p=0.05).

4.4.4 Effect of IBA on *in vitro* rooting of cowpea shoots

In vitro rooting of cowpea shoots was evaluated on MSB_5 medium containing different molar concentrations of IBA. The result indicated good rooting performance of the cowpea shoots both on IBA free and IBA supplemented medium. Except on medium containing 2-3 µM IBA, which gave 70-90 % rooting efficiencies, 100 % rooting efficiency was obtained on all other media (Table 12). The average number of roots per shoot ranged from 3.71 to 5.90 while the average root length ranged from 3.14 to 5.02 cm. In general, the addition of IBA into the medium did not significantly affect both the number of root and root length produced by the *in vitro* shoots. The *in vitro* rooted shoots were successfully acclimatized and transplanted to greenhouse (Fig. 30). The transplanted *in vitro* rooted plantlets were easily established under greenhouse with a success rate of 80%.

Table 12. Effect of IBA on *in vitro* rooting of cowpea shoots

MSB_5 + IBA (µM)	Rooting Efficiency (%)*	Root Number*	Root Length (cm)*
0	100[a]	5.40[a]	3.35[a]
1	100[a]	4.90[a]	4.31[a]
2	70[b]	3.71[a]	3.14[a]
2.5	70[b]	3.71[a]	4.14[a]
3	90[ab]	3.89[a]	3.37[a]
4	100[a]	5.30[a]	5.02[a]
5	100[a]	5.90[a]	3.85[a]
10	100[a]	4.80[a]	4.25[a]
15	100[a]	5.50[a]	4.55[a]
20	100[a]	4.70[a]	4.90[a]

*Means with the same letter within a column are not significantly different (REGWQ, p=0.05).

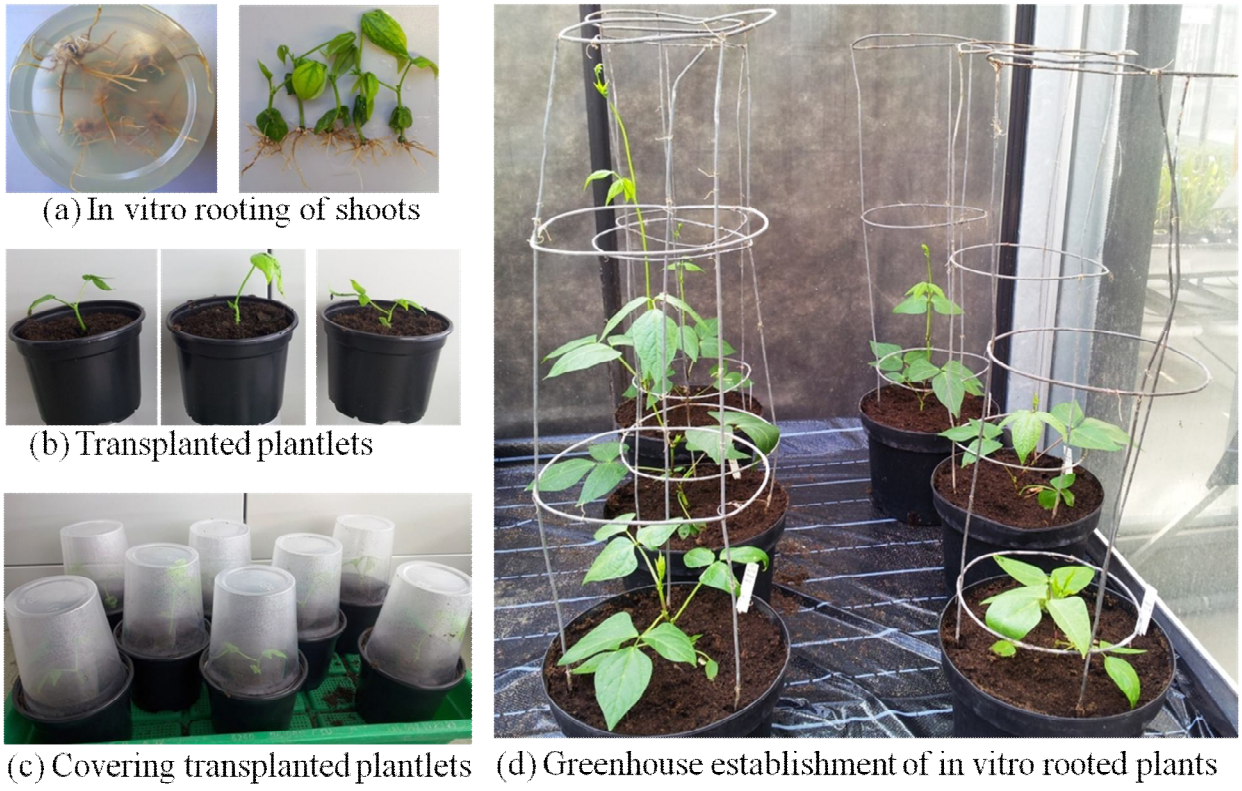


Fig. 30 Acclimatization of *in vitro* rooted cowpea shoots

4.4.5 Optimization of phosphinothricin (PPT) concentration for putative transgenic shoot selection

In order to selectively regenerate putative transgenic shoots, selection conditions were optimized on medium containing different concentrations of PPT (0-4.5 mg/L). Shoots survival was not observed on media containing 1.5 mg/L or more PPT (Table 13 and Fig. 31). About 97.1 and 77.6 % of the primary shoots survived on a medium without PPT and 0.5 mg/L PPT, respectively, while 100 and 82.7 % of the secondary shoots from CN survived on a medium without PPT and 0.5 mg/L PPT, respectively. The percentage of shoots surviving on a medium containing 1 mg/L PPT (4 % for primary shoots and 13.3 % for shoots from CN) was not significantly different from the result obtained on media containing 1.5 mg/L or more PPT.

Table 13. *In vitro* survival of shoots on MSB_5 medium supplemented with different concentration of phosphinothricin (PPT, mg/L)

PPT (mg/L)	Shoot survival (%)	
	Primary shoot from seeds*	Secondary shoots from CN*
0.0	97.1[a]	100[a]
0.5	77.6[b]	82.7[b]
1.0	4.0[c]	13.3[c]
1.5	0.0[c]	0.0[c]
2.0	0.0[c]	0.0[c]
2.5	0.0[c]	0.0[c]
3.0	0.0[c]	NA
3.5	0.0[c]	NA
4.0	0.0[c]	NA
4.5	0.0[c]	NA

*Means followed by the same letter within a column are not significantly different (REGWQ, p=0.05).
NA: Not applicable

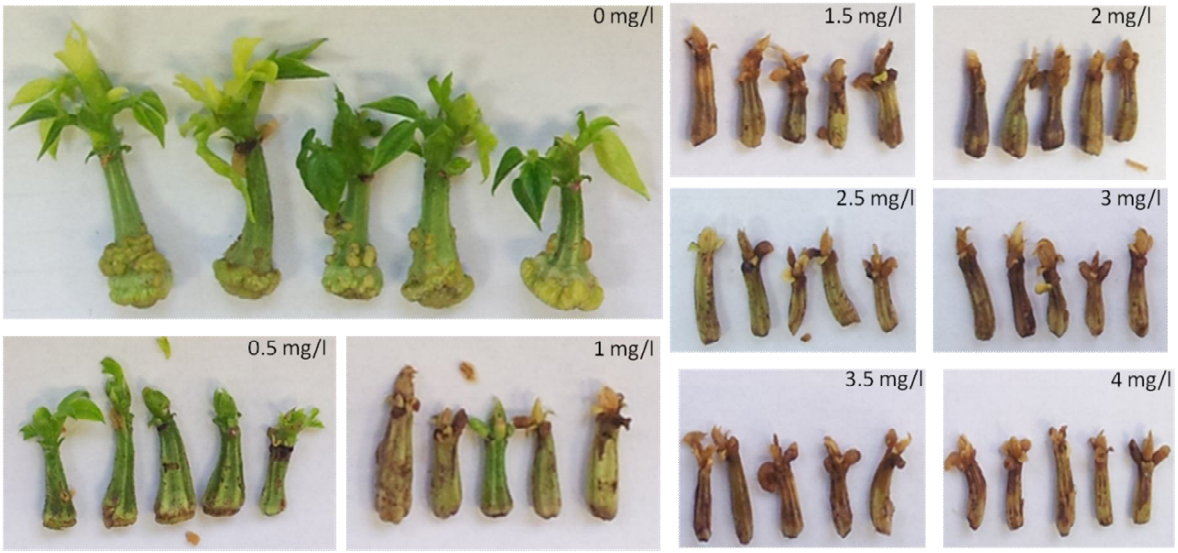


Fig. 31 State of primary shoots on medium supplemented with different concentration of phosphinothricin (PPT) after three weeks.

4.4.6 Optimization of inoculation and co-cultivation conditions using transient transformation

4.4.6.1 Effect of inoculation and co-cultivation media on transient transformation of DE and CN explants

Transient transformation was conducted using a transformation vector containing the *GUS* and *GFP* reporter genes in order to optimize inoculation and co-cultivation conditions. Three inoculation/co-cultivation media were tested. The result showed transient transformation efficiencies of 33-56.3 % and 22.6-84.5 % for *GUS* and *GFP* genes, respectively (Fig. 32 and 33). The highest transformation efficiency was obtained when CCMb medium was used for both *GUS* (56.3 %) and *GFP* (84.5 %) genes. The poorest transformation efficiency was observed on CCMc medium. Compared to embryo explants, very low transformation efficiencies were observed using CN explants in respective of the media used for inoculation and co-cultivation (1.92 % using CCMc, 3.28 % using CCMb and 6.52 % using B5i/B5hT).

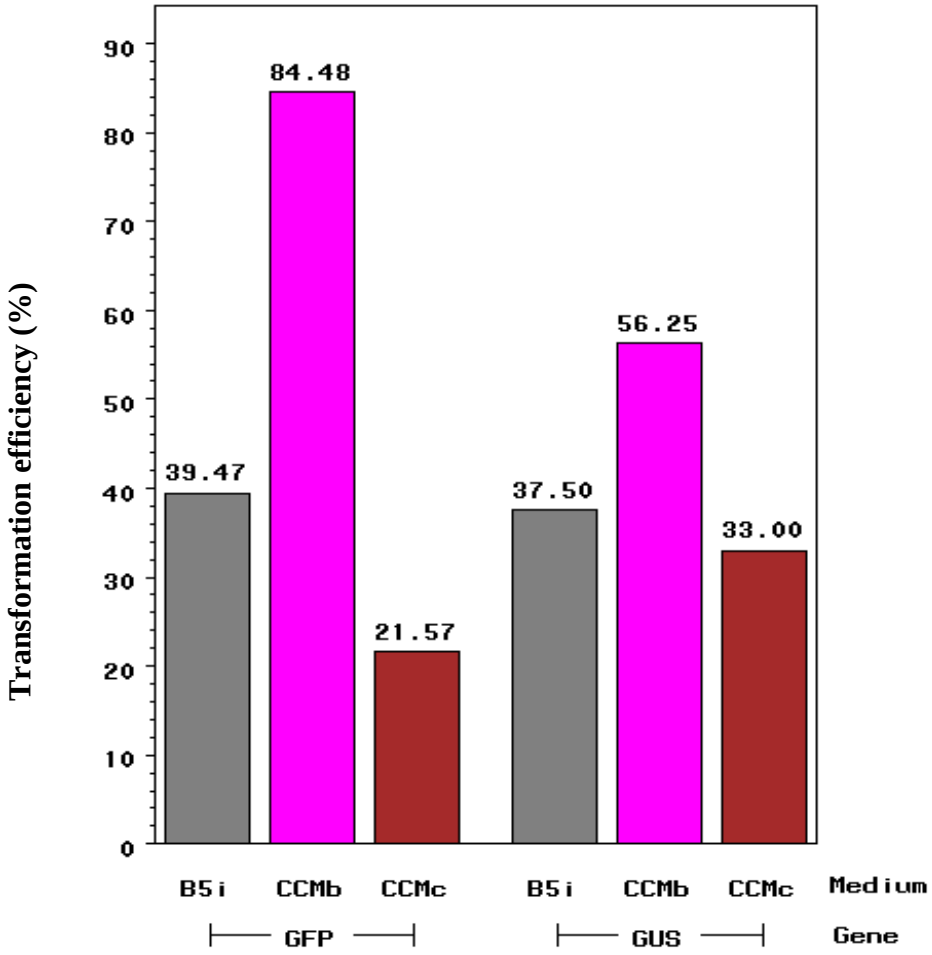


Fig. 32 Transient transformation efficiency in cowpea embryo from dry seeds using different inoculation and co-cultivation media.

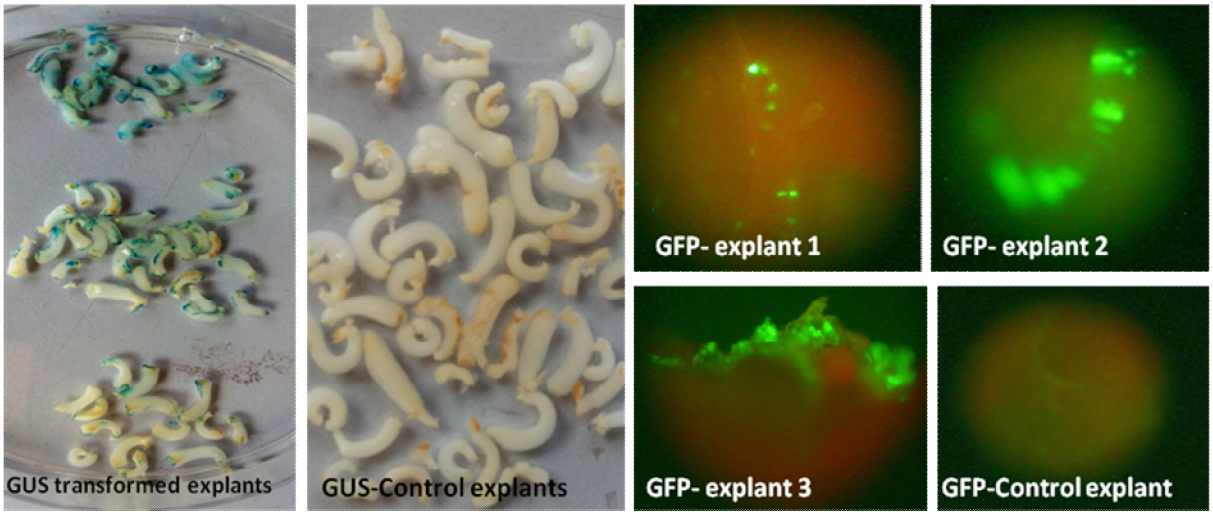


Fig. 33 Cowpea embryo explants with blue and green spot(s) showing the expression of *GUS* and *GFP* genes, respectively, and control explants.

The addition of sodium-thiosulphate and higher concentration of acetosyringone were reported to be very important for cowpea transformation (Popelka et al., 2006; Raveendar and Ignacimuthu, 2010). Accordingly, the CCMb inoculation/co-cultivation medium was modified by adding 1 mM sodium-thiosulphate and increasing the acetosyringone concentration from 100 µM to 200 µM [CCMbT]. A transformation vector containing the *GUS* gene was used. The result showed an improvement in the transformation efficiency of almost 40 % using the modified medium for inoculation/co-cultivation step (Fig. 34).

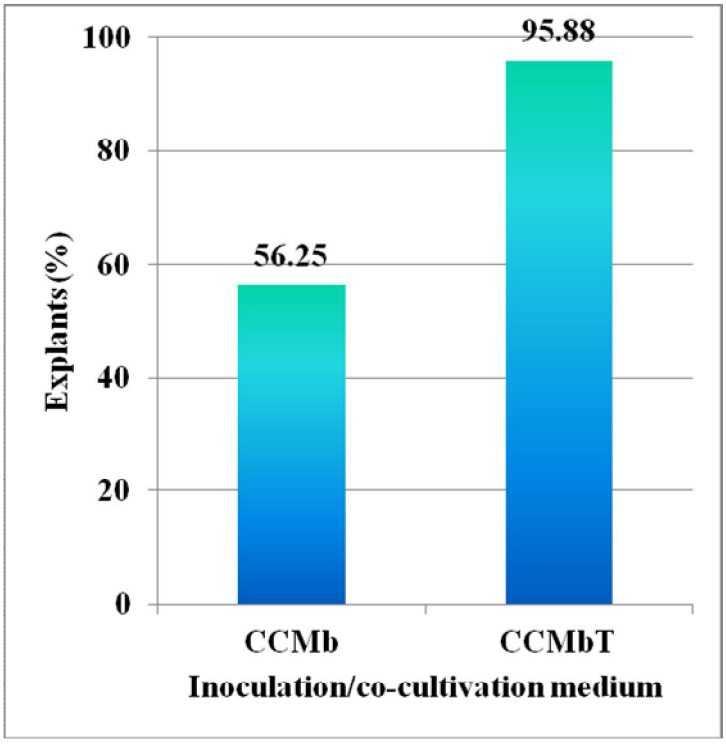


Fig. 34 Transient transformation efficiency as affected by addition of Na-thiosulphate and high concentration of acetosyringone into inoculation/co-cultivation medium. CCMb medium contain 100 µM acetosyringone and CCMbT medium contains 1mM Na-thiosulphate and 200 µM acetosyringone. The *Agrobacterium* suspension was adjusted to OD_{600} =1.

4.4.6.2 Sonication and vacuum infiltration assisted transient transformation in cowpea

With the aim of improving transformation efficiency, sonication and vacuum assisted transient transformation experiment was conducted with the *GUS* gene using the CCMb medium for inoculation and co-cultivation. The result showed no improvement in transient transformation when either sonication or vacuum infiltration or combination of both was used (Fig. 35). A transformation efficiency of 71.4 % was obtained when explants were not

treated with sonication and vacuum infiltration compared to 30.19-60.47 % when sonication and/or vacuum infiltration was used.

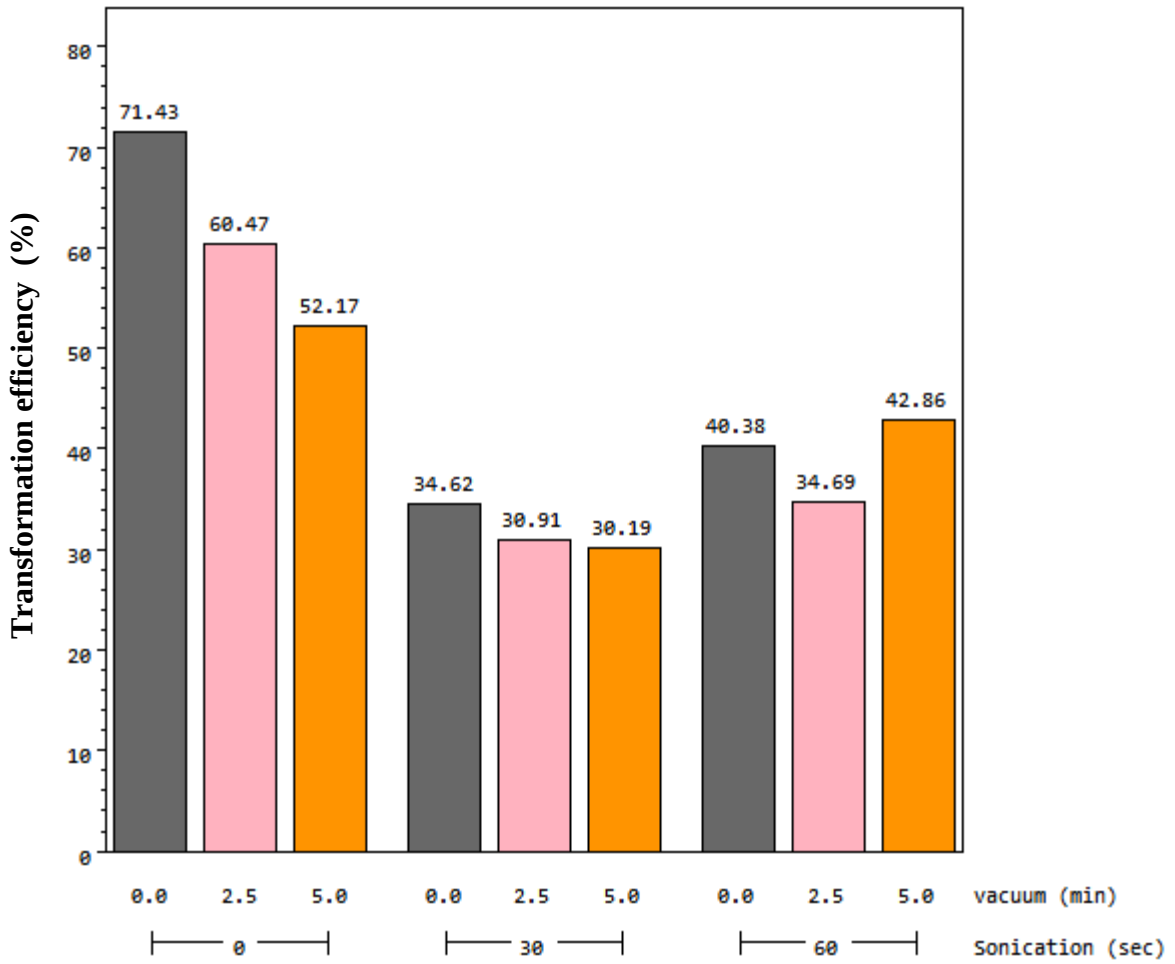


Fig. 35 Transient transformation efficiency of cowpea embryo slices using sonication and vacuum infiltration assisted *Agrobacterium* transformation

4.4.6.3 Effect of bacterial culture concentration on transient transformation

In addition to the media and explants, the effect of bacterial concentration on transient transformation efficiency was also tested using the *GUS* gene and DE explants. CCMbT (modified CCMb) medium was used for inoculation and co-cultivation step. Four bacterial concentrations (as determined at OD_{600} measurement: 0.5, 1, 1.5 and 2) and control were used. After co-cultivation, the GUS expression was detected and the explants were evaluated for transient transformation efficiency (TE, %) and the intensity of GUS expression. High transformation efficiencies of about 89 % to 99 % were observed

depending on the concentration of bacteria during inoculation. However, the intensity of GUS expression was not uniform across the concentration of bacteria used. Hence, based on the intensity of blue spots on the explant, transformed explants were categorized (visual assessment) into four groups: explants with strong, medium, weak and no GUS expression (Fig. 36). About 72 % of the explants showed medium to strong blue spots when a bacterial concentration of OD_{600}=2 was used compared to 32 %, 46 % and 54 % at OD_{600} of 0.5, 1.0 and 1.5, respectively (Fig. 37).

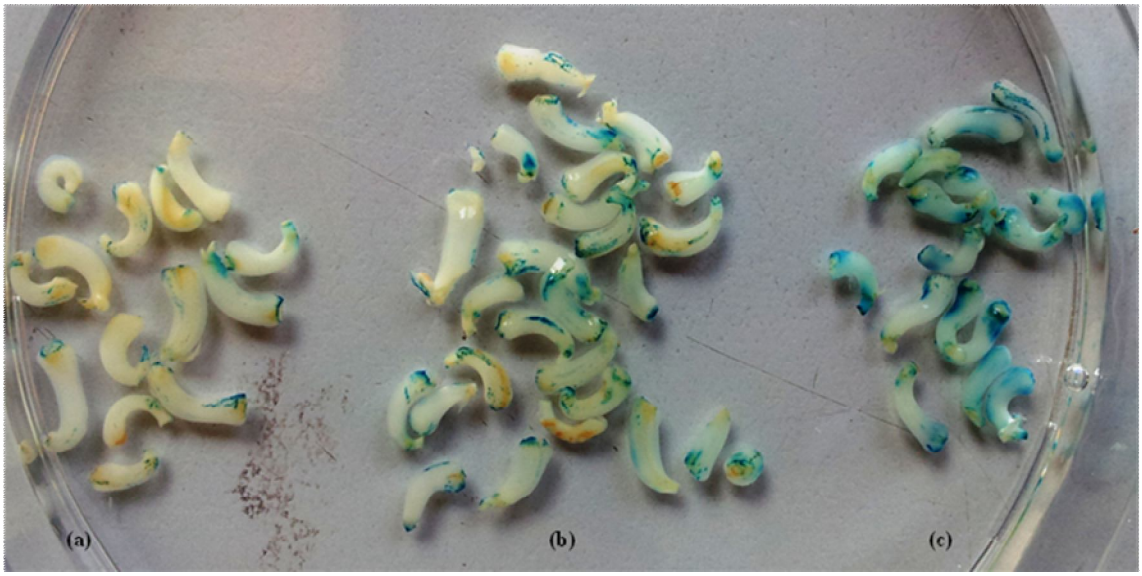


Fig. 36 Intensity of blue spot as a measure of *GUS* gene expression at OD_{600}=2.0 bacterial concentration. Explants with weak (a), medium (b) and strong (c) intensity of blue spots.

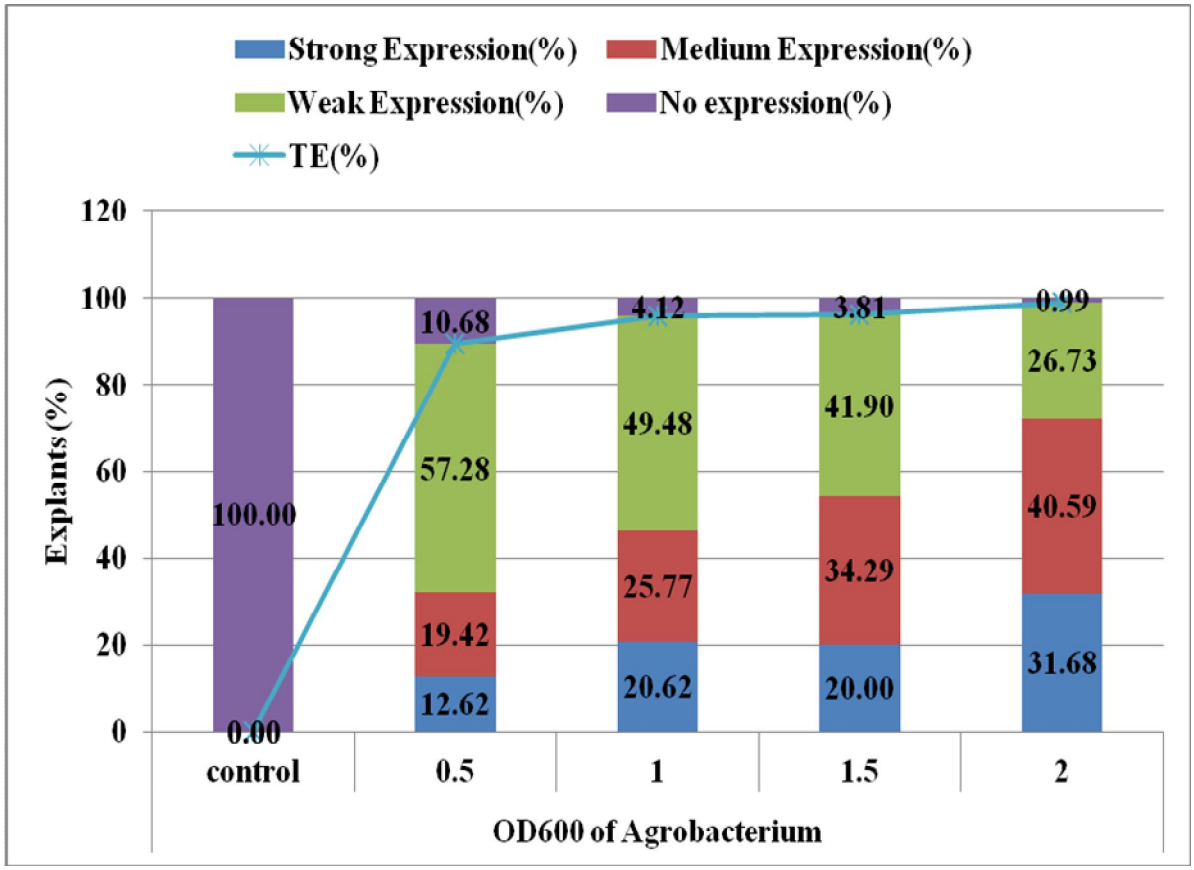


Fig. 37 Effect of *Agrobacterium* concentration (as determined at OD_{600} measurement) on transformation efficiency (TE, %) and the intensity of blue spot as a measure of GUS expression.

4.4.7 Transformation with *B.t cry* genes

Using the optimized protocol, experiments were conducted to introduce *Cry1Ac* and *Cry1Ab* genes for insect resistance into the genome of cowpea.

A number of transformation experiments were conducted with a vector containing the *cry* genes. More than 11 thousand explants (>9000 embryo explants from overnight soaked dry seeds and >2000 CN explants from a few days germinated seedlings) were treated (Table 14). A number of putative transgenic shoots were developed from the experiments. Some of the putative transgenic shoots were rooted and transferred to pots (Fig. 38) for further analysis of the transgene integration.

Table 14. Summary transformation experiments conducted with transformation vector harboring either *cry1Ac* or *cry1Ab* gene

Code	Variety	Transformation vector	Explant type	Number of explants (shoots)			Number of PCR positive shoots	Remark
				Co-cultivation	Shoot induction	survived after selection		
CP24	K80	PGII35S-Cry1Ab	ES	199	199	0		Browning*
CP25	K80	PGII35S-Cry1Ab	ES	220	220	0		Browning
CP27	K80	PGII35S-Cry1Ac	ES	430	407	0		Browning
CP28	K80	PGII35S-Cry1Ac	DE	409	-	-		
CP30	K80	PGII35S-Cry1Ac	CN	310	275	0		
CP31	K80	PGII35S-Cry1Ac	DE	143	130	0		
CP32	K80	PGII35S-Cry1Ac	DE	100	100	0		
CP33	K80	PGII35S-Cry1Ac	DE	109	109	0		
CP34	K80	PGII35S-Cry1Ac	CN	285	245	0		
CP35	K80	PGII35S-Cry1Ac	CN	230	-	-		
CP36	K80	PGII35S-Cry1Ac	DE	240	225	0		
CP37	K80	PGII35S-Cry1Ac	CN	180	180	0		
CP38	K80	PGII35S-Cry1Ac	CN	390	285	0		
CP39	K80	PGII35S-Cry1Ac	CN	373	270	0		
CP43	K80	PGII35S-Cry1Ac	ES	314	246	0		
CP45	K80	PGII35S-Cry1Ac	DE	176	161	0		
CP46	K80	PGII35S-Cry1Ac	ES	170	156	0		
CP47	K80	PGII35S-Cry1Ac	DE	187	184	0		
CP48	K80	PGII35S-Cry1Ac	DE	129	96	0		
CP49	K80	PGII35S-Cry1Ac	CN	45	-	-		Contamination
CP51	K80	PGII35S-Cry1Ab	DE	275	-	-		Contamination
Cp52	K80	PGII35S-Cry1Ac	DE	175	160	0		
CP54	K80	PGII35S-Cry1Ac	ES	170	170	0		
Cp55	K80	PGII35S-Cry1Ac	CN	220	220	0		
CP56	K80	PGII35S-Cry1Ac	DE	160	152	0		
Cp57	K80	PGII35S-Cry1Ac	ES	188	188	-		Contamination
Cp60	K80	PGII35S-Cry1Ac	DE	148	148	0		

*Browning was very common and most of the embryos failed to regenerate.

Table 14. Continuation

Code	Variety	Transformation vector	Explant type	Number of explants (shoots)			Number of PCR positive shoots	Remark
				Co-cultivation	Shoot induction	survived after selection		
Cp64	RB	PGII35S-Cry1Ac	DE	282	240	0		
Cp65	RB	PGII35S-Cry1Ac	DE	358	226	0		
Cp66	ICAP..	PGII35S-Cry1Ac	DE	196	190	0		
Cp67	VIT5	PGII35S-Cry1Ac	DE	39	14	0		
Cp68	K80	PGII35S-Cry1Ac	DE	110	?	2	0	
Cp69	K80	PGII35S-Cry1Ac	Callus	-	-	-		
Cp70	K80	PGII35S-Cry1Ac	DE	178	178	0		
Cp71	RB	PGII35S-Cry1Ab	DE	169	169	0		
Cp72	K80	PGII35S-Cry1Ab	DE & ES	380	380	0		
Cp74	K80	PGII35S-Cry1Ac	CN PN	150 144	150 144	0 0		Discarded**
Cp75	K80	PGII35S-Cry1Ac	DE	150	150	0		Discarded**
Cp76	K80	PGII35S-Cry1Ab	DE/EAC/DEP	418	258	4	0	
Cp77	K80	PGII35S-Cry1Ab	DEP	251	162	0		
Cp78	RB	PGII35S-Cry1Ac	DE	99	99	0		
Cp79	K80	PGII35S-Cry1Ac	DE	472	393	5	0	
Cp80	K80	PGII35S-Cry1Ac	DE	374	330	10	0	
Cp81	K80	PGII35S-Cry1Ac	DE	71	71	0		
Cp82	K80	PGII35S-Cry1Ac	DE	191	191	0		
Cp84	ICAPjJAG5773	PGII35S-Cry1Ac	EAC	82	82	0		
Cp85	Ethiopian/Gechi	PGII35S-Cry1Ac	DE	46	46	0		
Cp86	K80	PGII35S-Cry1Ac	DE	386	310	4	0	
Cp87	K80	PGII35S-Cry1Ab	DE	235	197	0		
Cp88	K80	pIBGUS	DE	124	124	1	0	
Cp89	K80	PGII35S-Cry1Ac	DE	246	246	8	2	
C90	K80	PGII35S-Cry1Ac	DE	332	332	0		

**Discarded due to regeneration problem.

Fig. 38 Few of the transplanted putative transgenic cowpea plantlets

4.4.8 PCR detection of transgene integration into the genome of putative transgenic cowpea plants

Putative transgenic shoots of cowpea were analyzed to determine the genomic integration of the transgene. Primers for *cry (cry1Ac* or *cry1Ab)* and *bar* genes were used for amplification of T-DNA region specific sequences. Primers for a cowpea housekeeping gene (cHMG) (Phelps et al., 2007) were used as an internal control while plasmid DNA was used as a positive control during PCR detection. Fig. 39 shows the PCR results of the putative transgenic shoots (*in vitro* shoots or transplanted plantlets) of cowpea from different transformation experiments. The expected PCR product was amplified for the housekeeping gene indicating the presence of genomic DNA in the reaction tube while the presence of the expected PCR product in the reaction tube containing plasmid DNA (+C) shows the PCR program is working. The expected PCR product of the *cry1Ac* transgene (T-DNA region) was observed in the reaction tubes containing the genomic DNA from two *in vitro* putative transgenic shoots (lane 29 and 30, Fig. 39 a). The expected PCR product was also observed using primers for *bar* gene for these two samples (Fig. 39 b). No amplification product was observed in the reaction tubes containing genomic DNA from the rest of the analyzed samples. The same transformation vector was used to transform pea where a number of transgenic lines were obtained (lane 23-35, Fig. 39 a). PCR analysis of *Agrobacterium* persistence showed absence of *Agrobacterium* specific DNA in the two PCR positive putative transgenic shoots (Fig. 39 c).

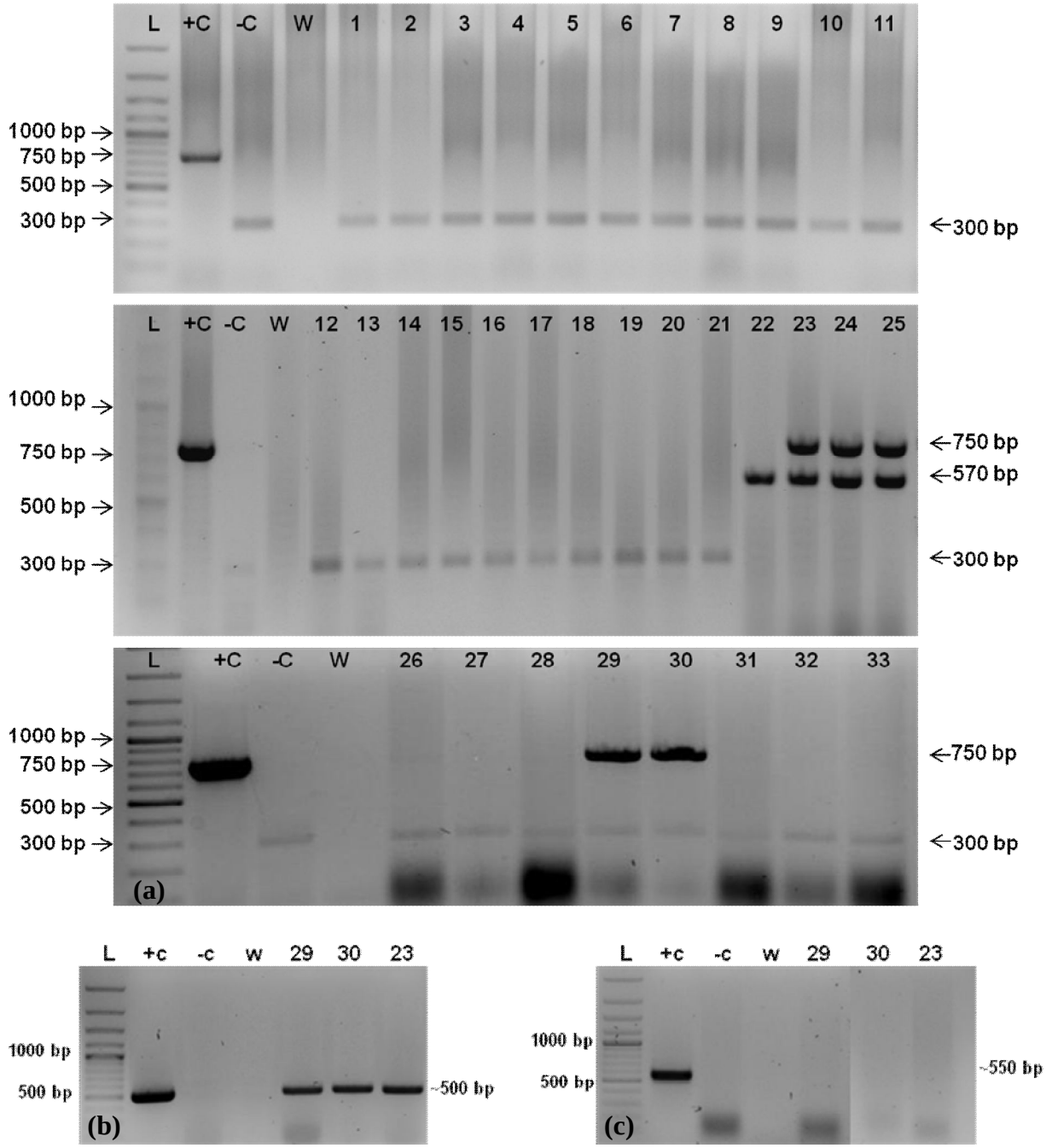
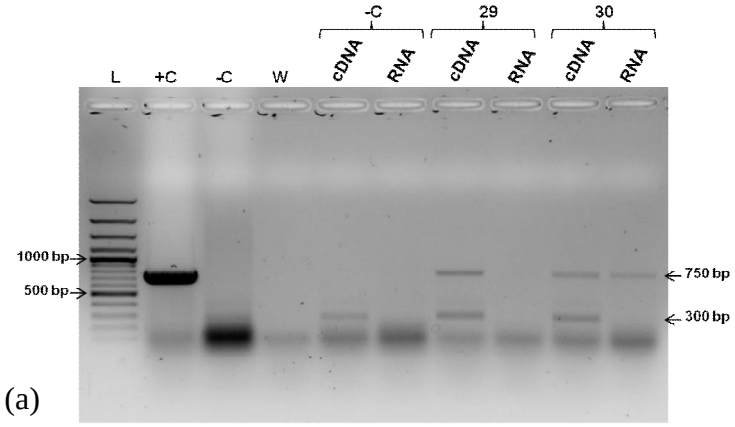


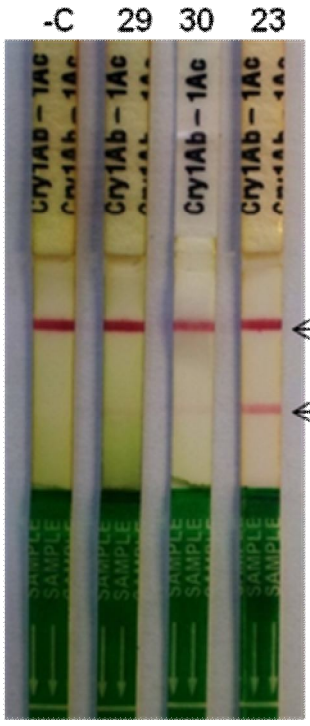
Fig. 39 PCR analysis of transgene integration into the genome of regenerated putative transgenic shoots of cowpea from different transformation experiments. Putative transgenic shoots of cowpea (variety K80) analyzed using primers for (a) *cry1Ac* transgene (~750 bp) and cowpea housekeeping gene (cHMG, ~ 300 bp), (b) *bar* gene (500 bp) and (c) *Agrobacterium* specific DNA sequences. L: GeneRuler^{TM} 100 bp plus DNA ladder, +C: plasmid (pGII35S-cry1Ac) DNA as a positive control, -C: genomic DNA of non-transgenic cowpea plant as a negative control, W: water control, lane 1-21 and 26-33: genomic DNA from putative transgenic shoots of cowpea and lane 22-25: genomic DNA from pea plants as a control samples showing the success of transformation in pea using the same transformation vector (22: non-transgenic control plants and 23-25: Cry1Ac transgenic pea plants). For pea, in addition to primers for *cry1Ac* gene, primers for pea housekeeping gene (HMG-I/Y, ~570 bp) were also used to monitor the presence of genomic DNA during PCR amplification.

4.4.9 Expression of *Cry1Ac* gene in the primary transformants

The expression of *cry1Ac* gene at transcription and protein levels in the two PCR positive *in vitro* shoots was analyzed using RT-PCR and immunostrip assay, respectively, as described in the previous section. The result showed the expression of *cry1Ac* gene and the accumulation of the novel Cry1Ac protein in the regenerated shoots (Fig. 40). The intensity of the test signal for the Cry1Ac protein in the two cowpea samples was relative weak as compared to that of the transgenic pea line used as a positive control.



(a)



Legend:

-C: Control cowpea plants

29 and 30: Putative transgenic cowpea shoots

23: Cry1Ac transgenic pea line as a positive control

(b)

Fig. 40 Expression of *cry1Ac* gene in the putative transgenic cowpea shoots. (a) RT-PCR analyis of *cry1Ac* gene at transcription level and (b) immunostrip detection of Cry1Ac protein.

4.4.10 Sensitivity of cowpea leaves to BASTA® herbicide solutions

In addition to the *in vitro* shoot sensitivity test on PPT containing media, leaf paint assay was also conducted on greenhouse grown non-transgenic plants to determine the concentration of herbicide solution to be used for leaf paint characterization of progenies from any transgenic lines. Basta® solutions (0-1000 mg/L) were applied on the upper side of the leaves and sensitivity of the leaves was evaluated one week after application. The result showed that the leaves of cowpea plants showed a little sign of necrosis when 50 mg/L herbicide solution was used (Fig. 41). The leaves showed partial wilting when 100 mg/L or more was used with completely wilting of the treated leaf starting from 300 mg/L. Based on this result, Basta® herbicide solution at a concentration of 300 mg/L could be used to characterize putative transgenic plants and their progeny during functional characterization (Fig. 42).

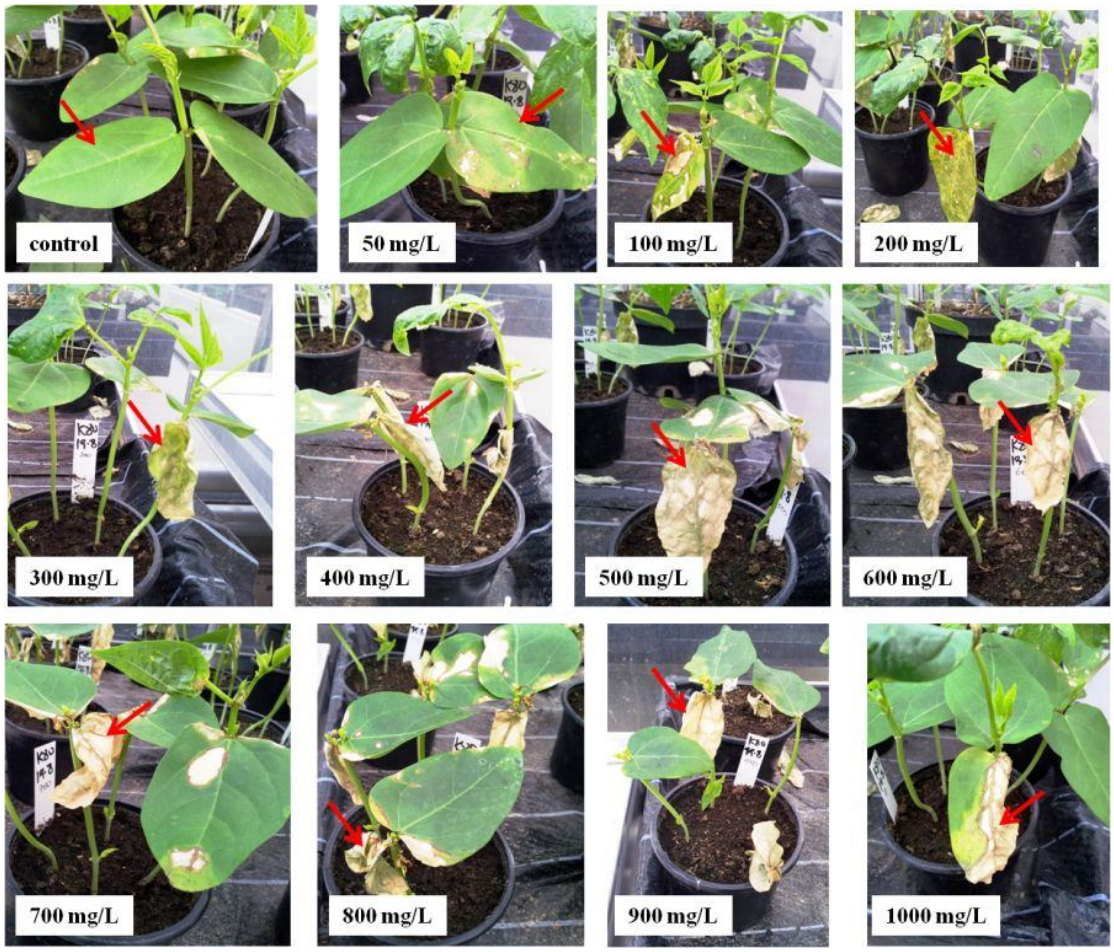


Fig. 41 Sensitive of non-transgenic cowpea leaves to Basta® herbicide solution at different concentration of active ingredient evaluated one week (at least) after application. The red arrows indicate the herbicide solution treated leaves.

Fig. 42 One of the putative transgenic plants characterized with leaf paint assay using 300 mg/L Basta® herbicide solution showing a negative result (red arrow) for the *bar* gene product.

4.5 Discussion

4.5.1 Optimization of *in vitro* conditions for regeneration and transformation of cowpea

4.5.1.1 Regeneration of Kenyan cowpea variety from CN explants

During the last three decades cowpea has been subjected to *in vitro* studies ranging from regeneration to genetic modification of traits via transgenic approaches. Despite these efforts, currently a universally applicable and genotype neutral regeneration and transformation protocol is lacking in cowpea. This has been long explained by the recalcitrant nature and varietal dependence of cowpea to *in vitro* manipulations (Brar et al., 1997b; Somers et al., 2003). This has limited the application of existing protocols for a variety of interest.

In the present study, *in vitro* conditions were optimized for a Kenyan cowpea variety (K80) for regeneration and transformation. Accordingly, a medium supplemented with 3 µM BA and 0.5 µM kinetin was found to be optimal for multiple shoots induction (4.8 shoots per CN explant). In earlier reports, different concentrations of BA either alone or in combination with other plant hormones (mostly kinetin) were used for multiple shoot production (1.14 to 13.5 shoots per CN explants) of cowpea (Mao et al., 2006; Popelka et al., 2006; Chaudhury et al., 2007; Solleti et al., 2008a; Raveendar et al., 2009; Bakshi et al., 2011; Aasim et al., 2012). The use of a range of BA concentration might be due to the wide cowpea gene pool and/or

the type of explants used by the different groups. Response variation among cowpea varieties and genotypes to *in vitro* regeneration conditions has been reported by different authors (Brar et al., 1999; Popelka et al., 2006; Raveendar et al., 2009). In addition, different types of explants (such as CN, decapitated embryo, shoot tips, plumular apices) were used in cowpea regeneration with varying rate of multiple shoot production per explants (Popelka et al., 2006; Chaudhury et al., 2007; Diallo et al., 2008; Raji et al., 2008; Solleti et al., 2008b; Yusuf et al., 2008; Aasim et al., 2009a, b; Raveendar et al., 2009; Aasim et al., 2010; Aasim et al., 2012; Bakshi et al., 2012a). These different types of explant might require different optimal *in vitro* conditions.

4.5.1.2 Effect of pre-conditioning media on multiple shoot production of CN explants

CN explants are commonly used in cowpea *in vitro* regeneration. For CN explant preparation, seeds are imbibed overnight and pre-conditioned (germinated) for 3-4 days. While sterile distilled water is used for overnight imbibitions of seeds, medium containing 10 µM BA or TDZ is commonly used for pre-conditioning of seeds (Chaudhury et al., 2007; Solleti et al., 2008a; Solleti et al., 2008b; Bakshi et al., 2011; Bakshi et al., 2012b; Bakshi and Sahoo, 2013). Van Le et al. (2002) had tested different concentration of TDZ (0, 1, 5, 10, 20, 50 µM) in pre-conditioning medium and found that more number of buds was produced by explants conditioned on medium containing 10 µM TDZ. Bakshi et al. (2012a) also studied the effect of both TDZ and BA (0, 5, 10 and 20 µM) as a pre-conditioning medium supplement and reported maximum number shoots per explants from CN explants obtained from a pre-conditioning medium containing 10 µM TDZ.

In line with this gap, pre-conditioning media supplemented with either BA or TDZ were tested for their effects on the multiple shoot production ability of CN explants. The result indicated that the use of moderate to high concentration of TDZ (2.5 -15 µM) had significantly reduced the number of shoots per explant. This is not in agreement with other reports (Van Le et al., 2002; Bakshi et al., 2012a) which showed 10 µM TDZ as the best pre-conditioning medium supplement. Moreover, the acceptable performance of CN explants

from a pre-conditioning medium with no growth hormone would be interesting from a developing countries point of view where cowpea is economically very important crop. [1]

4.5.1.3 *In vitro* rooting of cowpea shoots

Cowpea shoots were easily rooted on medium with or without IBA with no significant difference among the media. The result is in line with other reports which indicated the addition of IBA into rooting media had no effect on the rooting characteristics of *in vitro* shoots in cowpea (Mao et al., 2006; Tang et al., 2012). Hormone free rooting medium has been used in many cases of *in vitro* rooting of cowpea shoots (Muthukumar et al., 1995; Brar et al., 1999; Choi et al., 2003; Raji et al., 2008; Raveendar et al., 2009; Tie et al., 2013). Despite the acceptable rooting efficiency of the *in vitro* shoots of cowpea on hormone free media, rooting medium supplement with 2.5 µM IBA has been used in many cowpea regeneration and transformation studies (Chaudhury et al., 2007; Diallo et al., 2008; Solleti et al., 2008a; Solleti et al., 2008b; Aasim et al., 2009a, b, 2010; Bakshi et al., 2011; Aasim et al., 2012; Bakshi et al., 2012b; Aasim et al., 2013; Bakshi and Sahoo, 2013).

4.5.1.4 Sensitivity of cowpea to PPT

In plant transformation, one of the challenges faced by practitioners is the selection of cells with the introduced transgene of interest. This step is facilitated by co-introducing marker gene (with the gene of interest) encoding resistance/tolerance to selection agents (antibiotics or herbicides) (Miki and McHugh, 2004; Finer and Dhillon, 2008; Miki, 2008). In the transformation vectors used in this study, *bar* gene (Murakami et al., 1986; Thompson et al., 1987) was used for this purpose. It is one of the commonly used plant selectable marker genes in plant transformation (Miki and McHugh, 2004; Miki, 2008). Hence, the transformation process was aided using the herbicide active ingredient, PPT. The result of *in*

[1] In many developing countries, the affordability and availability of most of the plant growth hormones from reliable source is one of the challenges for the application of tissue culture in crop improvement. Thus, plant growth hormone free medium can be used for seedling pre-conditioning and the limitedly available growth hormone could be used for other activities.

vitro experiment demonstrated that PPT concentration as low as 1 mg/L was enough to kill most of the shoots within three weeks. Kononowicz et al. (1997) also found 1 mg/L bialaphos as effective concentration to kill explants (cotyledon, embryonic axes and plantlets) of cowpea. Theoretically; it is possible to use 1 mg/L PPT as the first selection pressure. However, in order to reduce the intensity of double stresses due to the selection agent and other *in vitro* conditions, 0.5 mg/L PPT was chosen as the first selection pressure for explants from transformation experiments and then increases the concentration at each subculture. On other hand, Popelka et al. (2006) have reported 4-6 mg/L PPT as the minimal concentration for effective selection of shoots for cowpea cultivar Sasaque. Adesoye and colleagues (Adesoye et al., 2010) also used 5 mg/L PPT for selection of putative transgenic shoots for cowpea variety IT96D-734. For the Turkish cowpea cultivar Akkiz, putative transgenic shoots were selected on a medium containing 2.5 mg/L PPT (Aasim et al., 2013). In this study, since there was no shoot survival on a medium containing 1.5 mg/L or higher concentration of PPT, the use of such elevated concentration of PPT could pose too much stress on the explants and thereby decreases the chance of getting surviving putative transgenic shoots. In addition, different cultivars of a crop species might have different levels of tolerance to selection agents in the medium (Galun and Breiman, 1998). Hence, it might be possible that cowpea cultivars have different levels of tolerance to PPT in the medium as well.

4.5.1.5 Effect of inoculation/co-cultivation media and explant on regeneration and transient transformation of cowpea

Of the inoculation and co-cultivation media tested using transient GUS and GFP expression, the highest transformation efficiency was obtained using a medium reported by Solleti et al. (2008a) compared to the other tested media. The addition of Na-thiosulphate (1 mM) and a high concentration of acetosyringone (200 μM) to this inoculation/co-cultivation medium has substantially improved the transformation efficiency. Similar observations have been reported in soybean where the addition of thiol compounds (Na-thiosulphate, dithiothreitol and L-Cysteine) in the co-cultivation medium has enhanced stable transformation (Olhoft et al., 2001; Olhoft and Somers, 2001; Olhoft et al., 2003).

In *Agrobacterium*-mediated transformation, the use of explants which have good regeneration efficiency as well as easily susceptible to *Agrobacterium* infection (competence for foreign

DNA uptake) is very vital in order to introduce the gene of interest into the plant genome. In most cowpea organogenesis experiments, explants such as CN, DE, EAC, sliced embryos and shoot tips were used while leaf cuttings are used for somatic embryogenesis. In the current study, both CN and DE explants showed a good regeneration efficiency. However, transient transformation efficiency using the *GUS* gene was very poor with CN explants as compared to DE explants. As a result, DE explants were selected for further transformation experiments.

During transformation, explants are wounded to make access for *Agrobacterium* infection and sometimes the transformation is assisted by sonication and vacuum infiltration (Finer and Dhillon, 2008). These procedures could promote browning of the damaged tissue and then determinately affect the regeneration ability of the explants. In some crops like pea, embryo slices (3-5 per seed) can be prepared without substantially affecting shoot regeneration ability. In other crops like cowpea, slicing/wounding of explants and application of sonication/vacuum infiltration negatively affect the regeneration of explants (Fig. 43). Most of the sliced/wounded explants failed to produce shoots. Furthermore, the shoot production ability of explants (even without wounding) seemed to decrease after transformation. This could be due to the presence of the bacteria (during the inoculation and co-cultivation steps) and the conditions (media and media ingredients) which could negatively affect the regeneration ability of the explants.

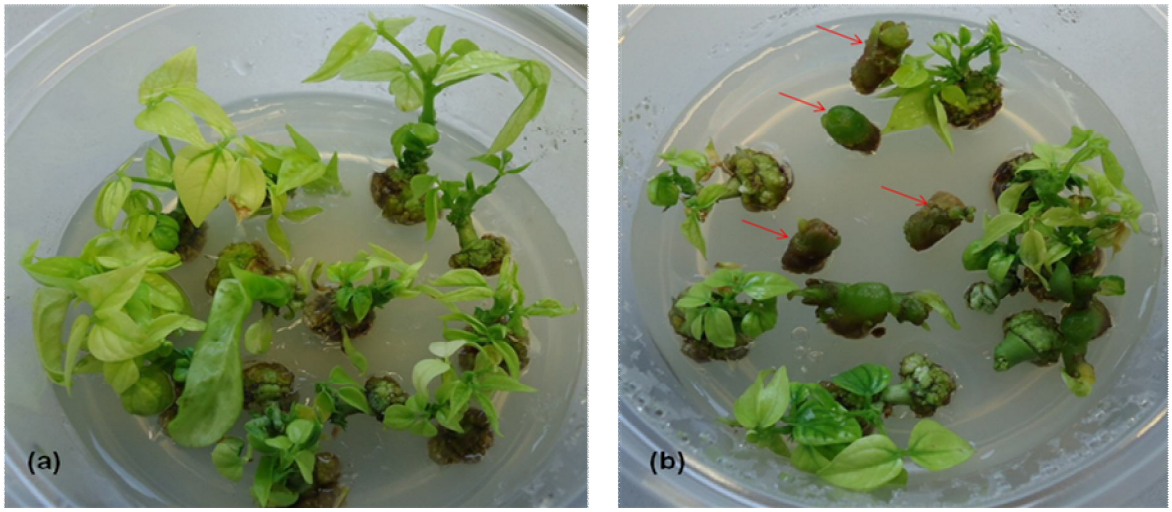


Fig. 43 Comparative regeneration of unwounded (a) and wounded (b) CN explants after three weeks of culturing on shoot induction medium. Wounded CN explants produced 1.32 shoots per explant with 78 % regeneration efficiency while intact unwounded CN explants produced 2.36 shoots per explant with 100 % regeneration efficiency. The arrows shows wounded CN explants that failed to produce shoots.

The successful development of transgenic plants depends on the ability to introduce the target gene into a totipotent and transformation competent area (cells and/or tissues) of the explants (Finer and Dhillon, 2008). This could be further complicated by different *in vitro* factors. For example, during the GUS detection of explants, it was observed that some of the explants showed strong GUS expression on hypocotyl region (root forming side) while others showed strong expression in the epicotyl region (shoot forming side). For this reason, transient transformation was conducted to test whether this is related to the explant orientation on co-cultivation medium. Three explant orientations (vertical upright plating by inserting the basal end into the media, inverted vertical plating by inserting the epicotyl end into the medium and horizontal plating) during co-cultivation were tested. The result showed that in majority of the explants, the *GUS* gene was expressed on the explant end that was not in contact with medium (Fig. 44, Appendix 8). Similar observation was reported in soybean in which the *GUS* gene expression on the explant tissue was strongly influenced by the orientation of the explants during co-cultivation (Ko et al., 2003). The transfer of T-DNA to plant cell is limited by factors affecting *vir* gene induction such as pH, culture aeration and presence of cell metabolites (Stachel et al., 1986). It is suggested that localized high pH and limited aeration on the side of explants in contact with the medium could inhibit the *vir* gene induction and then result in low or no expression of *GUS* gene (Santarem et al., 1998). In order to understand clearly the reason behind this localized expression of the *GUS* gene in the explants, besides *Agrobacterium* related factors, it is also very vital to understand the physiology and biology of explants during *in vitro* regeneration in general and co-cultivation in particular. According to Stachel and colleagues (Stachel et al., 1986), the constitutive presence of cell metabolites are very important for the induction of the *vir* genes. The explant side that faces away from (i.e., not in contact with) the medium might be engaged in active cell division and metabolism which then contributes to the presence of metabolites required for induction of *vir* genes (Santarem et al., 1998). The DNA replication process in actively dividing cell accelerates the integration of transgene into the genome (Finer and Dhillon, 2008). The result from this study suggested that, during co-cultivation, explants have to be cultured in vertical upright orientation in order to increase the chance of *Agrobacterium* infection and introduction of T-DNA region into shoots producing area of the explants.

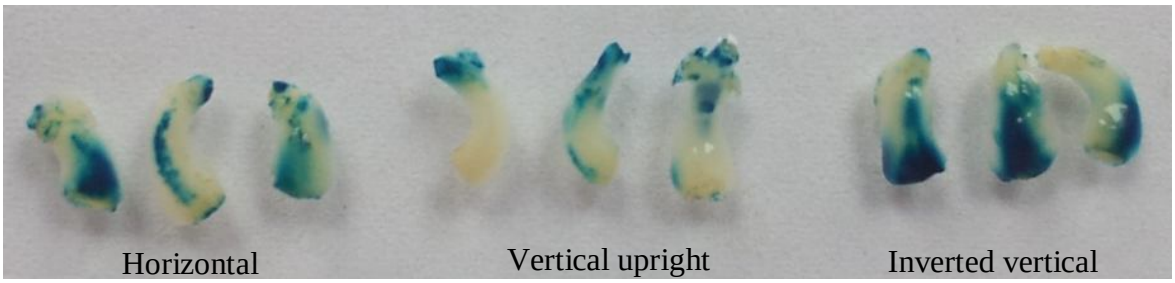


Fig. 44 Effect of explant orientations (horizontal, vertical upright and inverted vertical) during co-cultivation on *GUS* gene expression area of the explant.

4.5.1.6 Effect of *Agrobacterium* concentration of transient transformation of cowpea

In the effort to improve the transformation and increase the chance of getting stable transformation, the effect of *Agrobacterium* concentration (as measured by OD_{600}) in the inoculation medium was tested. Using around 2.0 (OD_{600}) in a medium containing a high concentration of acetosyringone (200 μM) and 1 mM Na-thiosulphate, consistently high transient transformation has been achieved. Hence, after harvesting, adjusting the OD_{600} of overnight culture to higher value might be more effective to increase the chance of introducing gene of interest into the plant cell.

4.5.2 Summary of the optimized protocol

In general, based on the optimized conditions, the transformation protocol can be summarized as shown in Table 15.

Table 15. Outline of transformation steps and time requirement

Steps	Media and solutions required	Time required	Days
Explant preparation			1-2
• Seed disinfection	Tap water 70 % Ethanol 6 % NaOCl Sterile distilled water (3-5 times rinsing)	~10 min 1 min 30 min ~ 10 mi	
• Seed imbibitions	Sterile distilled water	Overnight (10-12 hrs)	
• Embryo (DE) isolation		3-4 hr	
Inoculation	*Agrobacterium* suspended in inoculation medium (CCMbT medium: MSB + 1 µM BA + 1mM Na-thiosulphate + 8.3 mM L-cysteine + 200 µM acetosyringone + 1 g/L MES + 1 mM Dithiothreitol + 3 % sucrose)	60 min	2
Co-cultivation	CCMbT medium solidified with 7.5 g/L plant agar and plat the explant in vertical upright orientation	3 days	2-5
Collecting and Washing of co-cultivated explants	Explant collection Sterile distilled water (3-5 times rinsing) Antibiotic solution (Ticarcillin, 100 mg/L)	~30 min ~15 min 30 min on shaker	5
Shoot induction	Shoot induction medium (C_3K medium: MSB + 3 µM BA + 0.5 µM Kin + 1 g/L MES + 100 mg/L Ticarcillin + 100 mg/L Sulbactam)	3 weeks	Week 2-4
Putative transgenic shoot (PTS) selection	C_3K medium supplemented with PPT • 0.5 - 2 mg/L PPT	3 weeks each	Week 5-16
PTS elongation	C_3K medium	3 weeks	Week 17-20
Rooting	MSB with or with IBA (2.5 µM)	3 weeks	Week 21-24
Total time from explant preparation to rooting step			**~24 weeks**

4.5.3 Transformation with *B.t cry* genes

Using the optimized *in vitro* conditions, attempts were made to introduce *B.t cry* genes (*cry1Ac* or *cry1Ab*) into the genome of the selected Kenyan cowpea variety. Despite thousands of explants were transformed in a series of experiments, stable transformation was rarely achieved during the study time. Only two PCR positive primary transformants (*in vitro* shoots) were obtained in one of the experiments. They were obtained from 246 transformed explants giving a transformation efficiency of 0.81 %. This could be explained by the

recalcitrant nature of the crop (Brar et al., 1997b) that places it among the difficult to transform plant species with which only little success has been obtained so far.

In addition, transgene introduction and stable integration into the plant genome is affected by many factors such as the *Agrobacterium* strain used, the *in vitro* conditions (media composition, selection system, culture conditions, explant orientation, etc), the plant genotype, the explant type and the ability of delivering the transgene into regeneration competent cells/tissue of the explants (Altpeter, 2007). Though optimization was done, it might be possible that one or more of these factors have contributed to the lack of stable transformation in almost all of the experiments. The *Agrobacterium* strains EHA101 (containing pIBGUS) and EHA105 (containing pEGAD-GFP, in which the *cry* genes were also located) were used for transient transformation. The expression of the *GUS* (56%) and *GFP* (84 %) genes was observed on majority of the explants indicating the susceptibility of the explants and the effectiveness of the two strains for the transformation of the cowpea variety used in this study. Despite a decisive factor for successful transformation of plants (Grant and Cooper, 2006), lack of stable transformation could happen in the presence effective *Agrobacterium* strains. For example, the low rate of transgenic soybean plants recovery from co-cultured cotyledonary explants was not due to the poor susceptibility to *Agrobacterium* but by the inefficient transformation of regenerable cells and/or poor selection or survival of these cells (Donaldson and Simmonds, 2000). According to them, despite explant transformation rates of 27-92 %, transformation events were usually restricted to the non-regenerable callus of the explant. Eapen (2008) also suggested low transformation competency of regenerating cells as one of the reasons for the poor efficiency in transgenic grain legume development. For example, detection of GUS expression in callus and leaf parts of the *in vitro* shoots showed positive results on the callus part, but very rarely on the leaves (Fig. 45). Similarly, PCR analysis of *cry1Ac* integration in genomic DNA from callus and leaf parts of *in vitro* shoots showed few positive results in DNA from callus (Fig. 46), but not from leaf in most of the cases. Hence, in addition to other factors, the lack of regeneration from transformation competent areas of the explants could also be the reason why there was no successful stable transformation.

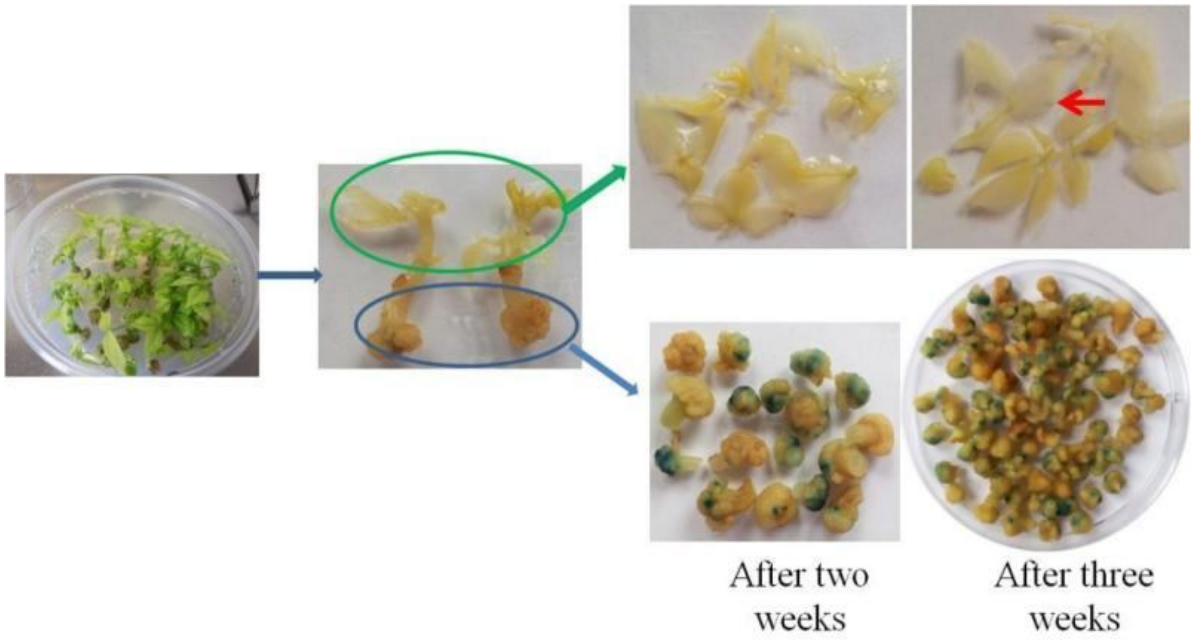


Fig. 45 GUS detection in the callus and leaf parts of the explants after 2-3 weeks culture on shoot induction medium.

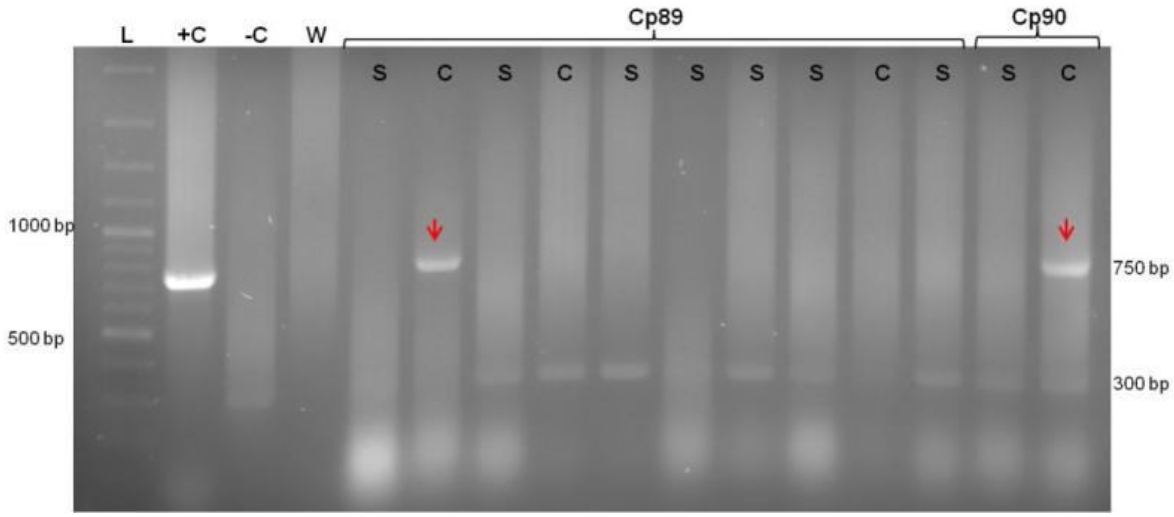


Fig. 46 PCR detection of cry1Ac gene in genomic DNA isolated from callus (C) and leaves (S) parts of explants surviving on selection medium.

4.5.4 Future outlook

In general, regeneration and stable transformation of cowpea is far from being a routine procedure and is still a challenging process due to different factors (Brar et al., 1997b; Somers et al., 2003; Chaudhury et al., 2007; Bakshi et al., 2011). Every working group has their own protocol for *in vitro* culture and transformation. This underlines the need to further explore *in vitro* conditions which could work across laboratories and genotypes. Based on the result from this study, in order to increase the chance of obtaining transgenic shoots, the following recommendations have been drawn in future efforts of cowpea transformation:

- Use of embryo explants from fresh and clean seeds collected from healthy mother plants, preferably grown under controlled conditions.
- Seeds infested with storage pests should be avoided since the *in vitro* return from such seeds is very minimal.
- Adjusting the concentration of *Agrobacterium* suspension to higher levels (OD_{600} around 2) might increase the chance of getting stable integration.
- Place explants in vertical upright orientation during co-cultivation.
- Screen varieties that are most responsive to *in vitro* conditions.

In addition, given the recalcitrance of the crop to *in vitro* conditions (Brar et al., 1997b; Somers et al., 2003), optimizing and testing the reproducibility and efficiency of other transformation approaches such as *in planta* nodal electroporation (Chowrira et al., 1995; Chowrira et al., 1996) and direct injection of DNA into ovary/pollen tube (Zhou et al., 1983; Luo and Wu, 1988; Li et al., 2002; Shou et al., 2002) could play a vital role in the future development of transgenic lines with novel traits in cowpea. These *in planta* approaches have been used in grain legumes (including cowpea), soybean, cotton, maize and rice.

Finally, further analyses are required on the two PCR positive *in vitro* shoots (primary transformants) in order to confirm the stable integration, inheritance, expression as well as functionality of the transgene.

5. General discussion and future outlook

Despite billions of dollars spent on the control measures, a substantial amount of potential crop yield is lost due to insect pests every year (Ferry and Gatehouse, 2010; Sharma et al., 2010; Gatehouse et al., 2011). To feed the increasing world population in the current trend of climate change, not only potential productivity has to be increased but also yield losses have to be reduced as much as possible (Godfray et al., 2010). The application of transgenic approaches can help to ease some of the challenges (Yadav et al., 2013) through providing multiple benefits in agricultural production systems. It can complement conventional breeding in developing crop varieties with new traits which are not present in the gene pool of the crop of interest such as insect and disease resistance as well as improved nutritional qualities (Korth, 2008). The application of transgenic approaches can also help to reduce the amount of agrochemical and its side effect on the ecosystem. Globally, the adoption of transgenic technology in agriculture has not only reduced the amount of pesticide (8.8 %) and its environmental impacts (EIQ by 18.7%) but also helped to cut the release of greenhouse gas from farming areas (Barfoot and Brookes, 2014). According to recent meta-analysis on the impacts of GM crops, crop yields have increased by 21 % due to effective pest control and lower crop damage, and the amount and cost of pesticides have reduced by 37 % and 39 %, respectively, by using GM crops compared to non-GM crops (Klümper and Qaim, 2014).

Transgenic insect resistance is the second most popular trait in agricultural biotechnology next to herbicide resistance (James, 2013). Resistant lines of many crops have been developed against different insect pests ranging from field to storage pests. Today, most of the commercialized insect resistant transgenic crops such as cotton and maize express *B.t cry* genes specific to the Lepidopteran insect pests (James, 2013). In grain legumes, despite heavy attack of by this group of insect pest, *B.t cry* genes are not used at all or at early developmental stage.

The lack of commercial production and use of transgenic grain legumes is attributed to many factors. One of the main reasons is the poor efficiency in the development of transgenic lines due to the recalcitrance nature of grain legumes (Popelka et al., 2004; Eapen, 2008). In addition, most of the grain legumes are economically more important in less developed countries of the world (Christou, 1997; Eapen, 2008) where resources are limited to apply modern transgenic approaches in crop improvement. As a result, much attention has not been

given to grain legumes regeneration and transformation (Christou, 1997; Eapen, 2008). Similar to other GM crops, the development of transgenic grain legumes is also affected by wrong perception about transgenic crops in some part of the world (Eapen, 2008).

Given the socio-economic importance in general and the potential contribution in alleviating poverty and malnutrition in many countries of the world, the perspective of legumes has been changing during the last few years in terms of modern biotechnology application. As a consequence, protocols have been optimized for a number of legume species. In few legumes such as pea, such protocols have been used to produce transgenic lines for different production constraints. However, in many legume species, there are problems with the reproducibility of such protocols and transgenic lines are difficult to achieve (Chandra and Pental, 2003; Popelka et al., 2004). This problem needs to be addressed in coordinated manner (at different levels such as scientists, consumers, fund providers, policy maker, etc) so that a universally applicable robust protocol could be optimized for the legume species in question. In addition, the information and technical experiences from the successful transformed legumes species such as pea and soybean could help to address some of the problems faced in stable transformation of other recalcitrant legume species.

References

Aasim, M., Khawar, K. M. & Ozcan, S. 2009a. Comparison of shoot regeneration on different concentrations of thidiazuron from shoot tip explant of cowpea on Gelrite and Agar containing medium. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* 37**,** 89-93.

Aasim, M., Khawar, K. M. & Ozcan, S. 2009b. *In vitro* micropropagation from plumular apices of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Scientia Horticulturae,* 122**,** 468-471.

Aasim, M., Khawar, K. M. & Ozcan, S. 2010. Efficient *in vitro* propagation from preconditioned embryonic axes of Turkish cowpea (*Vigna Unguiculata* L.) cultivar Akkiz. *Archives of Biological Sciences,* 62**,** 1047-1052.

Aasim, M., Khawar, K. M. & Ozcan, S. 2013. Production of herbicide-resistant cowpea (*Vigna unguiculata* L.) transformed with the *bar* gene. *Turkish Journal of Biology,* 37**,** 472-478.

Aasim, M., Ozcan, S. F., Khawar, K. M. & Ozcan, S. 2012. Comparative studies on the competence of axillary shoot regeneration on unsliced and longitudinally sliced cotyledon nodes of *Vigna unguiculata*. *Turkish Journal of Botany,* 36**,** 281-287.

Acharjee, S., Sarmah, B. K., Kumar, P. A., Olsen, K., Mahon, R., Moar, W. J., Moore, A. & Higgins, T. J. V. 2010. Transgenic chickpeas (*Cicer arietinum* L.) expressing a sequence-modified *cry2Aa* gene. *Plant Science,* 178**,** 333-339.

Adesoye, A., Machuka, J. & Togun, A. 2008. *Cry1Ab* trangenic cowpea obtained by nodal electroporation. *African Journal of Biotechnology,* 7.

Adesoye, A. I., Togun, A. O. & Machuka, J. 2010. Transformation of cowpea (*Vigna unguiculata* L. Walp.) by *Agrobacterium* infiltration. *Appl Biosci,* 30**,** 1845-60.

Akella, V. & Lurquin, P. 1993. Expression in cowpea seedlings of chimeric transgenes after electroporation into seed-derived embryos. *Plant cell reports,* 12**,** 110-117.

Altpeter, F. 2007. Perennial ryegrass (*Lolium perenne* L.). *In:* Wang, K. (ed.) *Agrobacterium Protocols.* Humana Press.

Ambrose, M. 2008. Garden pea. *Vegetables II.* Springer.

Anand, R. P., Ganapathi, A., Anbazhagan, V. R., Vengadesan, G. & Selvaraj, N. 2000. High frequency plant regeneration via somatic embryogenesis in cell suspension cultures of cowpea (*Vigna unguiculata* L. Walp). *In Vitro Cellular & Developmental Biology. Plant,* 36**,** 475-480.

Armstrong, C. L., Parker, G. B., Pershing, J. C., Brown, S. M., Sanders, P. R., Duncan, D. R., Stone, T., Dean, D. A., Deboer, D. L., Hart, J., Howe, A. R., Morrish, F. M., Pajeau, M. E., Petersen, W. L., Reich, B. J., Rodriguez, R., Santino, C. G., Sate, S. J., Schuler, W., Sims, S. R., Stehling, S., Tarochione, L. J. & Fromm, M. E. 1995. Field

evaluation of European corn borer control in progeny of 173 transgenic corn events expressing an insecticidal protein from *Bacillus thuringiensis*. *Crop Science,* 35**,** 550-557.

Atkins, C. & Mc Smith, P. 1997. Genetic transformation and regeneration of legumes. *In:* Legocki, A., Bothe, H. & Pühler, A. (eds.) *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture.* Springer Berlin Heidelberg.

Bakshi, S., Roy, N. K. & Sahoo, L. 2012a. Seedling preconditioning in thidiazuron enhances axillary shoot proliferation and recovery of transgenic cowpea plants. *Plant Cell Tissue and Organ Culture,* 110**,** 77-91.

Bakshi, S., Sadhukhan, A., Mishra, S. & Sahoo, L. 2011. Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration. *Plant cell reports,* 30**,** 2281-2292.

Bakshi, S., Saha, B., Roy, N. K., Mishra, S., Panda, S. K. & Sahoo, L. 2012b. Successful recovery of transgenic cowpea (*Vigna unguiculata*) using the 6-phosphomannose isomerase gene as the selectable marker. *Plant cell reports,* 31**,** 1093-1103.

Bakshi, S. & Sahoo, L. 2013. How relevant is recalcitrance for the recovery of transgenic cowpea: Implications of selection strategies. *Journal of Plant Growth Regulation,* 32**,** 148-158.

Barfoot, P. & Brookes, G. 2014. Key global environmental impacts of genetically modified (GM) crop use 1996-2012. *GM crops & food,* 5**,** 149-60.

Barton, K. A., Whiteley, H. R. & Yang, N. S. 1987. *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to Lepidopteran insects. *Plant physiology,* 85**,** 1103-1109.

Bean, S. J., Gooding, P. S., Mullineaux, P. M. & Davies, D. R. 1997. A simple system for pea transformation. *Plant cell reports,* 16**,** 513-519.

Biddle, A. J. & Cattlin, N. D. 2001. *Pests and diseases of peas and beans-A Colour handbook,* Manson Publishing Ltd. London

Biradar, S. S., Sridevi, O. & Salimath, P. M. 2010. Genetic enhancement of chickpea for pod borer resistance through expression of CryIAc protein. *Karnataka Journal of Agricultural Sciences,* 22.

Birnboim, H. C. & Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research,* 7**,** 1513-23.

Bohorova, N., Zhang, W., Julstrum, P., McLean, S., Luna, B., Brito, R. M., Diaz, L., Ramos, M. E., Diaz, R., Estanol, P., Ordonez, R. V., Pacheco, M., Castillo, C. R., Salgado, M. & Hoisington, D. 1999. Transgenic tropical maize with *cryIAb* and *cryIAc* genes from microprojectile bombardment of immature embryos. *Plant Biotechnology and in Vitro Biology in the 21st Century,* 36**,** 109-113.

Brar, M. S., Al-Khayri, J. M., Morelock, T. E. & Anderson, E. J. 1999. Genotypic response of cowpea *Vigna unguiculata* (L.) to *in vitro* regeneration from cotyledon explants. *In Vitro Cellular & Developmental Biology-Plant,* 35**,** 8-12.

Brar, M. S., AlKhayri, J. M., Shamblin, C. E., McNew, R. W., Morelock, T. E. & Anderson, E. J. 1997a. *In vitro* shoot tip multiplication of cowpea (*Vigna unguiculata* L. Walp). *In Vitro Cellular & Developmental Biology-Plant,* 33**,** 114-118.

Brar, M. S., Anderson, E. J., Morelock, T. E. & McNew, R. W. 1997b. The effect of media constituents on *in vitro* culturing of cowpea (*Vigna unguiculata*) shoot tip and leaf disk explants. *Journal of the Arkansas Academy of Science,* 51.

Bravo, A., Gill, S. S. & Soberon, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon,* 49**,** 423-435.

Bravo, A., Likitvivatanavong, S., Gill, S. S. & Soberon, M. 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochem Mol Biol,* 41**,** 423-31.

Breitler, J. C., Cordero, M. J., Royer, M., Meynard, D., San Segundo, B. & Guiderdoni, E. 2001. The-689/+197 region of the maize protease inhibitor gene directs high level, wound-inducible expression of the *cry1B* gene which protects transgenic rice plants from stemborer attack. *Molecular Breeding,* 7**,** 259-274.

Cardoza, V. 2008. Tissue culture: The manipulation of plant development. *In:* Stewart, C. N. (ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications.* John Wiley & Sons, Inc., Hoboken, NJ, USA.

Carozzi, N. & Koziel, M. 1997. *Advances in insect control: The role of transgenic plants,* London, Taylor & Francis.

Chandra, A. & Pental, D. 2003. Regeneration and genetic transformation of grain legumes: An overview. *Current Science,* 84**,** 381-387.

Chaudhury, D., Madanpotra, S., Jaiwal, R., Saini, R., Kumar, P. A. & Jaiwal, P. K. 2007. *Agrobacterium tumefaciens*-mediated high frequency genetic transformation of an Indian cowpea (*Vigna unguiculata* L. Walp.) cultivar and transmission of transgenes into progeny. *Plant Science,* 172**,** 692-700.

Cheng, X. Y., Sardana, R., Kaplan, H. & Altosaar, I. 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 2767-2772.

Choi, P. S., Cho, D. Y. & Soh, W. Y. 2003. Plant regeneration from immature embryo cultures of *Vigna unguiculata*. *Biologia Plantarum,* 47**,** 305-308.

Chowrira, G. M., Akella, V., Fuerst, P. E. & Lurquin, P. F. 1996. Transgenic grain legumes obtained by *in planta* electroporation-mediated gene transfer. *Molecular biotechnology,* 5**,** 85-96.

Chowrira, G. M., Akella, V. & Lurquin, P. F. 1995. Electroporation-mediated gene transfer into intact nodal meristems *in planta*. Generating transgenic plants without *in vitro* tissue culture. *Molecular biotechnology,* 3**,** 17-23.

Christou, P. 1997. Biotechnology applied to grain legumes. *Field Crops Research,* 53**,** 83-97.

Christou, P. & Twyman, R. M. 2004. The potential of genetically enhanced plants to address food insecurity. *Nutrition Research Reviews,* 17**,** 23-42.

Chyi, Y.-S., Jorgensen, R. A., Goldstein, D., Tanksley, S. D. & Loaiza-Figueroa, F. 1986. Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. *Molecular and General Genetics MGG,* 204**,** 64-69.

Citadin, C. T., Cruz, A. R. R. & Aragao, F. J. L. 2013. Development of transgenic imazapyr-tolerant cowpea (*Vigna unguiculata*). *Plant cell reports,* 32**,** 537-543.

Citadin, C. T., Ibrahim, A. B. & Aragao, F. J. 2011. Genetic engineering in cowpea (*Vigna unguiculata*): History, status and prospects. *GM crops,* 2**,** 144-9.

Clement, S. L., Hardie, D. C. & Elberson, L. R. 2002. Variation among accessions of *Pisum fulvum* for resistance to pea weevil. *Crop Science,* 42**,** 2167-2173.

Clement, S. L., Wightman, J. A., Hardie, D. C., Bailey, P., Baker, G. & McDonald, G. 2000. Opportunities for integrated management of insect pests of grain legumes. *Linking Research and Marketing Opportunities for Pulses in the 21st Century,* 34**,** 467-480.

Credland, P. F. 1994. Bioassays with bruchid beetles - Problems and (some) solutions. *Stored Product Protection, Vols 1 and 2***,** 509-516.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. & Dean, D. H. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews,* 62**,** 807-+.

Cutler, S. R., Ehrhardt, D. W., Griffitts, J. S. & Somerville, C. R. 2000. Random GFP :: cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 3718-3723.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M. F., Oliva, N., Abrigo, E., Khush, G. S. & Datta, S. K. 1998. Constitutive and tissue-specific differential expression of the *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. *Theoretical and Applied Genetics,* 97**,** 20-30.

de Filippis, L. F. 2012. Breeding for biotic stress tolerance in plants. *In:* Ashraf, M., Öztürk, M., Ahmad, M. S. A. & Aksoy, A. (eds.) *Crop Production for Agricultural Improvement.* Springer Netherlands.

de Kathen, A. & Jacobsen, H. J. 1990. *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. *Plant cell reports,* 9**,** 276-279.

de Maagd, R. A., Bravo, A. & Crickmore, N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics,* 17**,** 193-199.

Diallo, M. S., Ndiaye, A., Sagna, M. & Gassama-Dia, Y. K. 2008. Plants regeneration from African cowpea variety (*Vigna unguiculata* L. Walp). *African Journal of Biotechnology,* 7**,** 2828-2833.

Diouf, D. 2011. Recent advances in cowpea (*Vigna unguiculata* L. Walp) "omics" research for genetic improvement. *African Journal of Biotechnology,* 10**,** 2803-2810.

Diouf, D. & Hilu, K. W. 2005. Microsatellites and RAPD markers to study genetic relationships among cowpea breeding lines and local varieties in Senegal. *Genetic Resources and Crop Evolution,* 52**,** 1057-1067.

Dita, M. A., Rispail, N., Prats, E., Rubiales, D. & Singh, K. B. 2006. Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. *Euphytica,* 147**,** 1-24.

do Rego, M. M., do Rego, E. R., Araujo, G. F. & Otoni, W. C. 2012. *In vitro* morphogenic potential of two genotypes of cowpea (*Vigna unguilata* L. Walp). *Vii International Symposium on in Vitro Culture and Horticultural Breeding,* 961**,** 411-418.

Donaldson, P. A. & Simmonds, D. H. 2000. Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. *Plant cell reports,* 19**,** 478-484.

Doyle, J. J. & Doyle, J. L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus,* 12**,** 13-15.

Eapen, S. 2008. Advances in development of transgenic pulse crops. *Biotechnology advances,* 26**,** 162-8.

Edwards, K., Johnstone, C. & Thompson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic acids research,* 19**,** 1349.

Edwards, O. & Singh, K. B. 2006. Resistance to insect pests: What do legumes have to offer? *Euphytica,* 147**,** 273-285.

Ehlers, J. D. & Hall, A. E. 1997. Cowpea (Vigna unguiculata L. Walp.). *Field Crops Research,* 53**,** 187-204.

Fatokun, C. A. 2002. Breeding cowpea for resistance to insect pests: attempted crosses between cowpea and *V. vexillata*. *In:* C.A, F., S.A, T., B.B, S., P.M, K. & M., T. (eds.) *Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the world cowpea conference III, held at the International Institute of Tropical Agriculture, IITA, Ibadan Nigeria 4–8 2000 IITA, Ibadan, Nigeria.*

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E. & Gresshoff, P. M. 2010. Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology,* 52**,** 61-76.

Ferry, N. & Gatehouse, A. M. R. 2010. Transgenic Crop Plants for Resistance to Biotic Stress. *In:* Kole, C., Michler, C., Abbott, A. & Hall, T. (eds.) *Transgenic Crop Plants.* Springer Berlin Heidelberg.

Finer, J. & Dhillon, T. 2008. Transgenic plant production. *In:* Stewart, C. N. (ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications.* John Wiley & Sons, Inc., Hoboken, NJ, USA.

Finnegan, J. & Mcelroy, D. 1994. Transgene inactivation - Plants fight back. *Bio-Technology,* 12**,** 883-888.

Fischhoff, D. A., Bowdish, K. S., Perlak, F. J., Marrone, P. G., McCormick, S. M., Niedermeyer, J. G., Dean, D. A., Kusano-Kretzmer, K., Mayer, E. J., Rochester, D. E., Rogers, S. G. & Fraley, R. T. 1987. Insect tolerant transgenic tomato plants. *Nat Biotech,* 5**,** 807-813.

Fondevilla, S. & Rubiales, D. 2012. Powdery mildew control in pea. A review. *Agronomy for Sustainable Development,* 32**,** 401-409.

Galun, E. & Breiman, A. 1998. *Transgenic plants: With an appendix on intellectual properties and commercialisation of transgenic plants by John Barton,* London, Imperial College Press.

Gamborg, O. L., Constabe.F & Shyluk, J. P. 1974. Organogenesis in callus from shoot apices of *Pisum sativum*. *Physiologia plantarum,* 30**,** 125-128.

Gamborg, O. L., Miller, R. A. & Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental cell research,* 50**,** 151-8.

Garcia, J. A., Hille, J. & Goldbach, R. 1986. Transformation of cowpea (*Vigna unguiculata*) cells with an antibiotic resistance gene using a Ti plasmid derived vector. *Plant Science,* 44**,** 37-46.

Garcia, J. A., Hille, J., Vos, P. & Goldbach, R. 1987. Transformation of cowpea (*Vigna unguiculata*) with a full length DNA copy of cowpea mosaic virus messenger-RNA. *Plant Science,* 48**,** 89-98.

Gatehouse, A. M. R., Ferry, N., Edwards, M. G. & Bell, H. A. 2011. Insect-resistant biotech crops and their impacts on beneficial arthropods. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 366**,** 1438-1452.

Gatehouse, A. M. R., Shi, Y., Powell, K. S., Brough, C., Hilder, V. A., Hamilton, W. D. O., Newell, C. A., Merryweather, A., Boulter, D. & Gatehouse, J. A. 1993. Approaches to insect resistance using transgenic plants. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 342**,** 279-286.

Gepts, P., Beavis, W. D., Brummer, E. C., Shoemaker, R. C., Stalker, H. T., Weeden, N. F. & Young, N. D. 2005. Legumes as a model plant family. Genomics for food and feed report of the cross-legume advances through genomics conference. *Plant physiology,* 137**,** 1228-1235.

Giller, K. E. 2001. *Nitrogen fixation in tropical cropping systems,* Wallingford, Oxon ; New York, CABI Pub.

Girijashankar, V., Sharma, H. C., Sharma, K. K., Swathisree, V., Prasad, L. S., Bhat, B. V., Royer, M., San Secundo, B., Narasu, M. L., Altosaar, I. & Seetharama, N. 2005. Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). *Plant cell reports,* 24**,** 513-522.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M. & Toulmin, C. 2010. Food Security: The Challenge of Feeding 9 Billion People. *Science,* 327**,** 812-818.

Graham, P. H. & Vance, C. P. 2003. Legumes: Importance and constraints to greater use. *Plant physiology,* 131**,** 872-877.

Grant, J. & Cooper, P. 2006. Peas (*Pisum sativum* L.). *In:* Wang, K. (ed.) *Agrobacterium Protocols.* Humana Press.

Gulbitti-Onarici, S., Zaidi, M. A., Taga, I., Ozcan, S. & Altosaar, I. 2009. Expression of *cry1Ac* in transgenic tobacco plants under the control of a wound inducible promoter (AoPR1) isolated from *Asparagus officinalis* to control *Heliothis virescens* and *Manduca sexta*. *Molecular biotechnology,* 42**,** 341-349.

Gupta, R., Webster, C. I. & Gray, J. C. 1997. The single-copy gene encoding high-mobility-group protein HMG-I/Y from pea contains a single intron and is expressed in all organs. *Plant Molecular Biology,* 35**,** 987-992.

Hassan, F. 2006. *Heterologous expression of a recombinant chitinase from Streptomyces olivaceoviridis ATCC 11238 in transgenic pea (Pisum sativum L.).* PhD, Department of Plant Biotechnology, Institute of Plant Genetics, Leibniz Universität Hannover.

Hassan, F., Meens, J., Jacobsen, H. J. & Kiesecker, H. 2009. A family 19 chitinase (Chit30) from *Streptomyces olivaceoviridis* ATCC 11238 expressed in transgenic pea affects the development of *T. harzianum in vitro*. *Journal of biotechnology,* 143**,** 302-308.

Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S. & Mullineaux, P. M. 2000. pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology,* 42**,** 819-32.

Hofte, H. & Whiteley, H. R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological reviews,* 53**,** 242-55.

Hood, E. E., Gelvin, S. B., Melchers, L. S. & Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research,* 2**,** 208-218.

Hood, E. E., Helmer, G. L., Fraley, R. T. & Chilton, M. D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTIBO542 outside of transfer DNA. *Journal of bacteriology,* 168**,** 1291-1301.

Ikea, J., Ingelbrecht, I., Uwaifo, A. & Thottappilly, G. 2003. Stable gene transformation in cowpea (*Vigna unguiculata* L. Walp) using particle gun method. *African Journal of Biotechnology,* 2**,** 211-218.

Indurker, S., Misra, H. S. & Eapen, S. 2007. Genetic transformation of chickpea (*Cicer arietinum* L.) with insecticidal crystal protein gene using particle gun bombardment. *Plant cell reports,* 26**,** 755-763.

Indurker, S., Misra, H. S. & Eapen, S. 2010. *Agrobacterium*-mediated transformation in chickpea (*Cicer arietinum* L.) with an insecticidal protein gene: Optimisation of different factors. *Physiology and Molecular Biology of Plants,* 16**,** 273-284.

Ivo, N. L., Nascimento, C. P., Vieira, L. S., Campos, F. A. & Aragao, F. J. 2008. Biolistic-mediated genetic transformation of cowpea (*Vigna unguiculata*) and stable Mendelian inheritance of transgenes. *Plant cell reports,* 27**,** 1475-83.

Iyer, L. M., Kumpatla, S. P., Chandrasekharan, M. B. & Hall, T. C. 2000. Transgene silencing in monocots. *Plant molecular biology,* 43**,** 323-346.

Jackai, L. E. N. & Daoust, R. A. 1986. Insect Pests of Cowpeas. *Annual Review of Entomology,* 31**,** 95-119.

Jaiwal, P. K. & Singh, R. P. 2003. *Improvement strategies of leguminosae biotechnology,* Boston, MA, Kluwer Academic Publishers.

James, C. 2013. Global status of commercialized Biotech/GM Crops: 2013. ISAAA Brief No. 46. ISAAA: Ithaca, NY.

Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. 1987. GUS fusions: *beta-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. *The EMBO journal,* 6**,** 3901-7.

Jia, S. 2004. *Transgenic cotton*, Elsevier.

Jouanin, L., Bonade-Bottino, M., Girard, C., Morrot, G. & Giband, M. 1998. Transgenic plants for insect resistance. *Plant Science,* 131**,** 1-11.

Kar, S., Basu, D., Das, S., Ramkrishnan, N. A., Mukherjee, P., Nayak, P. & Sen, S. K. 1997. Expression of *cryIA(c)* gene of *Bacillus thuringiensis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. *Transgenic Research,* 6**,** 177-185.

Kartha, K. K., Pahl, K., Leung, N. L. & Mroginski, L. A. 1981. Plant regeneration from meristems of grain legumes - Soybean, cowpea, peanut, chickpea, and bean. *Canadian Journal of Botany-Revue Canadienne De Botanique,* 59**,** 1671-1679.

Kefale, T. B. 2006. *Transformation of Ethiopian pea (Pisum sativum) varieties with cry1Ab and cry1Ac genes.* Master of Science, Department of Plant Biotechnology, Institute of Plant Genetics, Leibniz Universität Hannover.

Keneni, G., Bekele, E., Getu, E., Imtiaz, M., Damte, T., Mulatu, B. & Dagne, K. 2011. Breeding food legumes for resistance to storage insect pests: Potential and limitations. *Sustainability,* 3**,** 1399-1415.

Khanna, H. K. & Raina, S. K. 2002. Elite indica transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). *Transgenic Research,* 11**,** 411-423.

Klümper, W. & Qaim, M. 2014. A meta-analysis of the impacts of genetically modified crops. *PloS one,* 9**,** e111629.

Ko, T. S., Lee, S., Krasnyanski, S. & Korban, S. S. 2003. Two critical factors are required for efficient transformation of multiple soybean cultivars: *Agrobacterium* strain and orientation of immature cotyledonary explant. *Theoretical and Applied Genetics,* 107**,** 439-447.

Kohli, A., Melendi, P. G., Abranches, R., Capell, T., Stoger, E. & Christou, P. 2006. The quest to understand the basis and mechanisms that control expression of introduced transgenes in crop plants. *Plant signaling & behavior,* 1**,** 185.

Kohli, A., Miro, B. & Twyman, R. 2010. Transgene integration, expression and stability in plants: Strategies for improvements. *In:* Kole, C., Michler, C., Abbott, A. & Hall, T. (eds.) *Transgenic Crop Plants.* Springer Berlin Heidelberg.

Kononowicz, A. K., Cheah, K. T., Narasimhan, M. L., Murdock, L. L., Shade, R. E., Chrispeels, M. J. Z., Filippone, E., Monti, L. M., Bressan, R. A. & Hasegawa, P. M. 1997. Developing a transformation systems for cowpea (*Vigna unguiculata* L. Walp). *In:* Singh, B. B., Mohan Raj, D. R., Dashiell, K. E. & Jackai, L. E. N. (eds.) *Advances in Cowpea Research.* International Institute of Tropical Agriculture (UTA) and Japan International Research Center for Agricultural Sciences (JIRCAS). UTA, Ibadan, Nigeria.

Korth, K. L. 2008. Genes and traits of interest for transgenic plants. *In:* Stewart, C. N. (ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications.* John Wiley & Sons, Inc., Hoboken, NJ, USA.

Koul, B., Srivastava, S., Sanyal, I., Tripathi, B., Sharma, V. & Amla, D. V. 2014. Transgenic tomato line expressing modified *Bacillus thuringiensis cry1Ab* gene showing complete resistance to two lepidopteran pests. *SpringerPlus,* 3**,** 84.

Koutsika-Sotiriou, M. & Traka-Mavrona, E. 2008. Snap bean. *Vegetables II.* Springer.

Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., Mcpherson, K., Meghji, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M. & Evola, S. V. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio-Technology,* 11**,** 194-200.

Krejci, P., Matuskova, P., Hanacek, P., Reinohl, V. & Prochazka, S. 2007. The transformation of pea (*Pisum sativum* L.): Applicable methods of *Agrobacterium tumefaciens*-mediated gene transfer. *Acta Physiologiae Plantarum,* 29**,** 157-163.

Krishnamurthy, K. V., Suhasini, K., Sagare, A. P., Meixner, M., de Kathen, A., Pickardt, T. & Schieder, O. 2000. *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes. *Plant cell reports,* 19**,** 235-240.

Kulothungan, S., Ganapathi, A., Shajahan, A. & Kathiravan, K. 1995. Somatic embryogenesis in cell suspension culture of cowpea (*Vigna unguiculata* L. Walp). *Israel Journal of Plant Sciences,* 43**,** 385-390.

Kumari, M., Patade, V. Y. & Ahmad, Z. 2012. A simple, rapid and cost-effective method of DNA extraction from different plant species for a wide range of PCR based applications. *Vegetable Science,* 39**,** 118-122.

Legowski, T. J. & Gould, H. J. 1960. Losses of dry harvesting peas due to pea moth in East Anglia and the economics of control measures. *Plant Pathology,* 9**,** 119-126.

Lehminger-Mertens, R. & Jacobsen, H. J. 1989. Plant regeneration from pea protoplasts via somatic embyogenesis. *Plant cell reports,* 8**,** 379-82.

Li, J., Dai, X. B., Liu, T. S. & Zhao, P. X. 2012. LegumeIP: an integrative database for comparative genomics and transcriptomics of model legumes. *Nucleic acids research,* 40**,** D1221-D1229.

Li, Z., Nelson, R. L., Widholm, J. M. & Bent, A. 2002. Soybean transformation via the pollen tube pathway. *Soybean Genet Newslett,* 29**,** 1-11.

Lindsey, K. 1992. Genetic manipulation of crop plants. *Journal of biotechnology,* 26**,** 1-28.

Lucas, M., Ehlers, J., Huynh, B.-L., Diop, N.-N., Roberts, P. & Close, T. 2013. Markers for breeding heat-tolerant cowpea. *Molecular Breeding,* 31**,** 529-536.

Luo, Z.-x. & Wu, R. 1988. A simple method for the transformation of rice via the pollen-tube pathway. *Plant Molecular Biology Reporter,* 6**,** 165-174.

Machuka, J. 2002. Potential role of transgenic approaches in the control of cowpea insect pests. *In:* Fatokun, C. A., Tarawali, S. A., Singh, B. B., Kormawa, P. M. & Tamò, M. (eds.) *Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, 4–8 September 2000. IITA, Ibadan, Nigeria.*

Machuka, J., Adesoye, A. & Obembe, O. O. 2002. Regeneration and genetic transformation in cowpea. *In:* Fatokun, C. A., Tarawali, S. A., Singh, B. B., Kormawa, P. M. & Tamò, M. (eds.) *Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture (IITA), 2002 Ibadan, Nigeria.*

Mandaokar, A. D., Goyal, R. K., Shukla, A., Bisaria, S., Bhalla, R., Reddy, V. S., Chaurasia, A., Sharma, R. P., Altosaar, I. & Kumar, P. A. 2000. Transgenic tomato plants resistant to fruit borer (*Helicoverpa armigera* Hubner). *Crop Protection,* 19**,** 307-312.

Manman, T., Qian, L., Huaqiang, T., Yongpeng, Z., Jia, L. & Huanxiu, L. 2013. A review of regeneration and genetic transformation in cowpea (*Vigna unguiculata* L. Walp). *African Journal of Agricultural Research,* 8**,** 1115-1122.

Manoharan, M., Khan, S. & James, O. 2008. Improved plant regeneration in cowpea through shoot meristem. *J. App. Hortic,* 10**,** 40-43.

Mao, J. Q., Zaidi, M. A., Arnason, J. T. & Altosaar, I. 2006. *In vitro* regeneration of *Vigna unguiculata* (L.) Walp. cv. Blackeye cowpea via shoot organogenesis. *Plant Cell Tissue and Organ Culture,* 87**,** 121-125.

Mehrotra, M., Singh, A. K., Sanyal, I., Altosaar, I. & Amla, D. V. 2011. Pyramiding of modified *cry1Ab* and *cry1Ac* genes of *Bacillus thuringiensis* in transgenic chickpea (*Cicer arietinum* L.) for improved resistance to pod borer insect *Helicoverpa armigera*. *Euphytica,* 182**,** 87-102.

Meyer, P. 1995. Variation of transgene expression in plants. *Euphytica,* 85**,** 359-366.

Miki, B. 2008. Marker genes and promoters. *In:* Stewart, C. N. (ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications.* John Wiley & Sons, Inc., Hoboken, NJ, USA.

Miki, B. & McHugh, S. 2004. Selectable marker genes in transgenic plants: Applications, alternatives and biosafety. *Journal of biotechnology,* 107**,** 193-232.

Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K. & Thompson, C. J. 1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: Molecular cloning and characterization of the gene cluster. *Molecular & General Genetics,* 205**,** 42-50.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum,* 15**,** 473-497.

Muthukumar, B., Mariamma, M. & Gnanam, A. 1995. Regeneration of plants from primary leaves of cowpea. *Plant Cell Tissue and Organ Culture,* 42**,** 153-155.

Muthukumar, B., Mariamma, M., Veluthambi, K. & Gnanam, A. 1996. Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L. Walp) using *Agrobacterium tumefaciens*. *Plant cell reports,* 15**,** 980-985.

Negawo, A. T. 2012. *Transgenic insect resistance in pea (Pisum sativum L.).* MSc thesis, Department of Plant Biotechnology, Institute of Plant Genetics, Leibniz Universität Hannover.

Negawo, A. T., Aftabi, M., Jacobsen, H.-J., Altosaar, I. & Hassan, F. S. 2013. Insect resistant transgenic pea expressing *cry1Ac* gene product from *Bacillus thuringiensis*. *Biological Control,* 67**,** 293-300.

Nene, Y. L. & Reed, W. 1994. Integrated management systems to control biotic and abiotic stresses in cool season food legumes. *Expanding the Production and Use of Cool Season Food Legumes,* 19**,** 666-678.

Odukkathil, G. & Vasudevan, N. 2013. Toxicity and bioremediation of pesticides in agricultural soil. *Reviews in Environmental Science and Bio-Technology,* 12**,** 421-444.

Olhoft, P. M., Flagel, L. E., Donovan, C. M. & Somers, D. A. 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonary node method. *Planta,* 216**,** 723-735.

Olhoft, P. M., Lin, K., Galbraith, J., Nielsen, N. C. & Somers, D. A. 2001. The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary node cells. *Plant cell reports,* 20**,** 731-737.

Olhoft, P. M. & Somers, D. A. 2001. L-cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary node cells. *Plant cell reports,* 20**,** 706-711.

Ozcan, S., Barghchi, M., Firek, S. & Draper, J. 1992. High frequency adventitious shoot regeneration from immature cotyledons of pea (*Pisum sativum* L.). *Plant cell reports,* 11**,** 44-7.

Pajni, H. R. & Gill, M. 1991. Ecological status of store bruchids and its bearing on phylogeny. *In:* Fleurat-Lessard, F. & Ducom, P. (eds.) *Proceedings of the 5th International Working Conference on Stored Product Protection, 9-14 September 1990, Imprimerie du Médoc, Bordeaux, France.*

Parrott, W. A., All, J. N., Adang, M. J., Bailey, M. A., Boerma, H. R. & Stewart Jr, C. N. 1994. Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki insecticidal gene. *In Vitro Cellular & Developmental Biology-Plant,* 30**,** 144-149.

Penza, R., Akella, V. & Lurquin, P. F. 1992. Transient expression and histological localization of a *gus* chimeric gene after direct transfer to mature cowpea embryos. *BioTechniques,* 13**,** 576, 578, 580.

Penza, R., Lurquin, P. F. & Filippone, E. 1991. Gene transfer by cocultivation of mature embryos with *Agrobacterium tumefaciens*: Application to cowpea (*Vigna unguiculata* Walp). *Journal of plant physiology,* 138**,** 39-43.

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T. & Fischhoff, D. A. 1990. Insect resistant cotton plants. *Bio/technology,* 8**,** 939-43.

Phelps, J. P., Dang, N. & Rasochova, L. 2007. Inactivation and purification of cowpea mosaic virus-like particles displaying peptide antigens from *Bacillus anthracis*. *Journal of virological methods,* 141**,** 146-53.

Pniewski, T. & Kapusta, J. 2005. Efficiency of transformation of Polish cultivars of pea (*Pisum sativum* L.) with various regeneration capacity by using hypervirulent *Agrobacterium tumefaciens* strains. *J Appl Genet,* 46**,** 139-47.

Popelka, J. C., Gollasch, S., Moore, A., Molvig, L. & Higgins, T. J. 2006. Genetic transformation of cowpea (*Vigna unguiculata* L.) and stable transmission of the transgenes to progeny. *Plant cell reports,* 25**,** 304-12.

Popelka, J. C., Terryn, N. & Higgins, T. J. V. 2004. Gene technology for grain legumes: Can it contribute to the food challenge in developing countries? *Plant Science,* 167**,** 195-206.

Prols, F. & Meyer, P. 1992. The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia Hybrida*. *Plant Journal,* 2**,** 465-475.

Puonti-Kaerlas, J., Eriksson, T. & Engström, P. 1990. Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. *Theor. App1. Genet.,* 80**,** 246-252.

Raji, A. A. J., Oriero, E., Odeseye, B., Odunlami, T. & Ingelbrecht, I. L. 2008. Plant regeneration and *Agrobacterium*-mediated transformation of African cowpea (*Vigna unguiculata* L. Walp) genotypes using embryonic axis explants. *Journal of Food Agriculture & Environment,* 6**,** 350-356.

Ramakrishnan, K., Gnanam, R., Sivakumar, P. & Manickam, A. 2005. *In vitro* somatic embryogenesis from cell suspension cultures of cowpea (*Vigna unguiculata* L. Walp). *Plant cell reports,* 24**,** 449-461.

Ramesh, S., Nagadhara, D., Reddy, V. D. & Rao, K. V. 2004. Production of transgenic indica rice resistant to yellow stem borer and sap sucking insects using super binary vectors of *Agrobacterium tumefaciens. Plant Science,* 166**,** 1077-1085.

Ranalli, P. 2003. Breeding methodologies for the improvement of grain legumes. *In:* Jaiwal, P. K. & Singh, R. P. (eds.) *Improvement Strategies of Leguminosae Biotechnology.* Springer.

Raveendar, S. & Ignacimuthu, S. 2010. Improved *Agrobacterium*-mediated transformation in cowpea (*Vigna unguiculata* L. Walp). *Asian Journal of Plant Sciences,* 9**,** 256-263.

Raveendar, S., Premkumar, A., Sasikumar, S., Ignacimuthu, S. & Agastian, P. 2009. Development of a rapid, highly efficient system of organogenesis in cowpea (*Vigna unguiculata* L. Walp). *South African Journal of Botany,* 75**,** 17-21.

Richter, A., de Kathen, A., de Lorenzo, G., Briviba, K., Hain, R., Ramsay, G., Jacobsen, H. J. & Kiesecker, H. 2006. Transgenic peas (*Pisum sativum*) expressing polygalacturonase inhibiting protein from raspberry (*Rubus idaeus*) and stilbene synthase from grape (*Vitis vinifera*). *Plant Cell Reports,* 25**,** 1166-1173.

Rubatzky, V. E. & Yamaguchi, M. 1997. *World vegetables : Principles, production and nutritive values,* New York, Chapman & Hall : International Thomson Pub.

Saini, R. K. & Sharma, S. S. (eds.) 2013. *Advances in pest management in legumes crops,* Hisar-125 004 India: Centre of Advanced Faculty Training, Departemnt of Entomology, CCS Haryana Agricultural University. Hisar, India.

Santarem, E. R., Trick, H. N., Essig, J. S. & Finer, J. J. 1998. Sonication assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: Optimization of transient expression. *Plant cell reports,* 17**,** 752-759.

Sanyal, I., Singh, A. K., Kaushik, M. & Amla, D. V. 2005. *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis cry1Ac* gene for resistance against pod borer insect *Helicoverpa armigera. Plant Science,* 168**,** 1135-1146.

Sardana, R., Dukiandjiev, S., Giband, M., Cheng, X. Y., Cowan, K., Sauder, C. & Altosaar, I. 1996. Construction and rapid testing of synthetic and modified toxin gene sequences

CryIA (b&c) by expression in maize endosperm culture. *Plant cell reports,* 15**,** 677-681.

Sarmah, B. K., Moore, A., Tate, W., Molvig, L., Morton, R. L., Rees, D. P., Chiaiese, P., Chrispeels, M. J., Tabe, L. M. & Higgins, T. J. V. 2004. Transgenic chickpea seeds expressing high levels of a bean alpha-amylase inhibitor. *Molecular Breeding,* 14**,** 73-82.

Schroeder, H. E., Barton, J. E., Tabe, L. M., Molvig, L., Grant, J. E., Jones, M. & Higgins, T. J. V. 2000. Gene technology for improved weed, insect, and disease control and for seed protein quality. *Linking Research and Marketing Opportunities for Pulses in the 21st Century,* 34**,** 389-396.

Schroeder, H. E., Gollasch, S., Moore, A., Tabe, L. M., Craig, S., Hardie, D. C., Chrispeels, M. J., Spencer, D. & Higgins, T. J. V. 1995. Bean alpha amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). *Plant physiology,* 109**,** 1129-1129.

Schroeder, H. E., Schotz, A. H., Wardley-Richardson, T., Spencer, D. & Higgins, T. 1993. Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). *Plant physiology,* 101**,** 751-757.

Sebastian, K. T. 1983. Shoot tip culture and subsequent regeneration in cowpea. *Scientia Horticulturae,* 20**,** 315-317.

Shade, R. E., Schroeder, H. E., Pueyo, J. J., Tabe, L. M., Murdock, L. L., Higgins, T. J. V. & Chrispeels, M. J. 1994. Transgenic pea seeds expressing the alpha-amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio-Technology,* 12**,** 793-796.

Sharma, H. C. 2008. Insect pest management in food legumes: The future strategies. *In:* Kharkwal, M. C. (ed.) *Food legumes for nutritional security and sustainable agriculture, Proceedings of the IVth International Food Legumes Research Conference,18-22 Oct 2005.* Indian Society of Genetics and Plant Breeding, New Delhi, India.

Sharma, H. C., Sharma, K. K., Seetharama, N. & Ortiz, R. 2000. Prospects for using transgenic resistance to insects in crop improvement. *Electronic Journal of Biotechnology,* 3**,** 21-22.

Sharma, H. C., Srivastava, C. P., Durairaj, C. & Gowda, C. L. L. 2010. Pest management in grain legumes and climate change. *In:* Yadav, S. S., Redden, R., Mcneil, D. L. & Patil, S. A. (eds.) *Climate change and management of cool season grain legume crops.* Springer Netherlands.

Shou, H. X., Palmer, R. G. & Wang, K. 2002. Irreproducibility of the soybean pollen-tube pathway transformation procedure. *Plant Molecular Biology Reporter,* 20**,** 325-334.

Singh, B. B., Ehlers, J. D., Sharma, B. & Freire Filho, F. R. 2002. Recent progress in cowpea breeding. *In:* Fatokun, C. A., Tarawali, S. A., Singh, B. B., Kormawa, P. M. & Tamò, M. (eds.) *Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of*

Tropical Agriculture (IITA),Ibadan, Nigeria, 4–8 September 2000. Ibadan, Nigeria: IITA.

Singsit, C., Adang, M. J., Lynch, R. E., Anderson, W. F., Wang, A. M., Cardineau, G. & OziasAkins, P. 1997. Expression of a *Bacillus thuringiensis cryIA(c) gene* in transgenic peanut plants and its efficacy against lesser corn stalk borer. *Transgenic Research,* 6**,** 169-176.

Solleti, S. K., Bakshi, S., Purkayastha, J., Panda, S. K. & Sahoo, L. 2008a. Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean alpha-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles. *Plant cell reports,* 27**,** 1841-1850.

Solleti, S. K., Bakshi, S. & Sahoo, L. 2008b. Additional virulence genes in conjunction with efficient selection scheme, and compatible culture regime enhance recovery of stable transgenic plants in cowpea via *Agrobacterium tumefaciens*-mediated transformation. *Journal of biotechnology,* 135**,** 97-104.

Somers, D. A., Samac, D. A. & Olhoft, P. M. 2003. Recent advances in legume transformation. *Plant physiology,* 131**,** 892-9.

Stachel, S. E., Nester, E. W. & Zambryski, P. C. 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences of the United States of America,* 83**,** 379-383.

Stam, M., Mol, J. N. M. & Kooter, J. M. 1997. The silence of genes in transgenic plants. *Annals of botany,* 79**,** 3-12.

Stewart Jr, C. N., Adang, M. J., All, J. N., Boerma, H. R., Cardineau, G., Tucker, D. & Parrott, W. A. 1996. Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis cryIAc* gene. *Plant physiology,* 112**,** 121-129.

Tang, Y., Chen, L., Li, X., Li, J., Luo, Q., Lai, J. & Li, H. 2012. Effect of culture conditions on the plant regeneration via organogenesis from cotyledonary node of cowpea (*Vigna unguiculata* L. Walp). *African Journal of Biotechnology,* 11**,** 3270-3275.

Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M. & Botterman, J. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *The EMBO journal,* 6**,** 2519-23.

Tie, M., Luo, Q., Zhu, Y. & Li, H. 2013. Effect of 6-BA on the plant regeneration via organogenesis from cotyledonary node of cowpea (*Vigna unguiculata* L. Walp). *Journal of Agricultural Science,* 5.

Timko, M. P., Ehlers, J. D. & Roberts, P. A. 2007. Cowpea. *In:* Kole, C. (ed.) *Pulses, Sugar and Tuber Crops, Genome Mapping and Molecular Breeding in Plants.* Berlin Heidelberg: Springer-Verlag.

Timko, M. P. & Singh, B. B. 2008. Cowpea, a multifunctional legume. *In:* Moore, P. H. & Ming, R. (eds.) *Genomics of Tropical Crop Plants.* New York , NY Springer Science + Business Media, LLC.

Tzitzikas, E. N., Bergervoet, M., Raemakers, K., Vincken, J. P., van Lammeren, A. & Visser, R. G. 2004. Regeneration of pea (*Pisum sativum* L.) by a cyclic organogenic system. *Plant cell reports,* 23**,** 453-60.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Montagu, M. V. & Leemans, J. 1987. Transgenic plants protected from insect attack. *Nature,* 328**,** 33-37.

Van Le, B., de Carvalho, M. H. C., Zuily-Fodil, Y., Thi, A. T. P. & Van, K. T. T. 2002. Direct whole plant regeneration of cowpea (*Vigna unguiculata* L. Walp) from cotyledonary node thin cell layer explants. *Journal of plant physiology,* 159**,** 1255-1258.

Varshney, R. K., Close, T. J., Singh, N. K., Hoisington, D. A. & Cook, D. R. 2009. Orphan legume crops enter the genomics era! *Current Opinion in Plant Biology,* 12**,** 202-210.

Wallroth, M., Gerats, A. G. M., Rogers, S. G., Fraley, R. T. & Horsch, R. B. 1986. Chromosomal localization of foreign genes in *Petunia hybrida*. *Molecular and General Genetics MGG,* 202**,** 6-15.

Williams, L., Schotzko, D. J. & Okeeffe, L. E. 1995. Pea leaf weevil herbivory on pea seedlings - Effects on growth response and yield. *Entomologia Experimentalis Et Applicata,* 76**,** 255-269.

Yadav, R. C., Solanke, A. U., Kumar, P., Pattanayak, D., Yadav, N. R. & Kumar, P. A. 2013. Genetic Engineering for Tolerance to Climate Change-Related Traits. *In:* Kole, C. (ed.) *Genomics and Breeding for Climate-Resilient Crops.* Springer.

Young, N. D. & Bharti, A. K. 2012. Genome enabled insights into legume biology. *Annual Review of Plant Biology, Vol 63,* 63**,** 283-305.

Young, N. D., Mudge, J. & Ellis, T. H. N. 2003. Legume genomes: More than peas in a pod. *Current Opinion in Plant Biology,* 6**,** 199-204.

Yusuf, M., Raji, A., Ingelbrecht, I. & Katung, M. 2008. Regeneration efficiency of cowpea (*Vigna unguiculata* L. Walp) via embryonic axes explants. *African Journal of Plant Science,* 2**,** 105-108.

Zhou, G.-Y., Weng, J., Zeng, Y., Huang, J., Qian, S. & Liu, G. 1983. Introduction of exogenous DNA into cotton embryos. *Methods in Enzymology,* 101**,** 433-481.

Appendices

Appendix 1. List of equipment

Autoclave (Tuttnauer systec)

Balances (Sartorius)

Cold centrifuge (Sigma 302K)

Cryogenic vials (Cryoware-Nalgene, Rochester, USA)

Deep freezer -80°C (Lozone)

Dry oven (Memmert)

Electrophoresis chamber (Bio-RAD)

Electrophoresis power supply (Bio-RAD)

Ice machine (ZIEGRA)

Incubator (JURGENS)

Lab centrifuge (Eppendorf 5415C)

Magnetic stirrer (Heidolph)

Microwave (Thomson)

pH meter (Hanna)

Pipette (Gilson, Eppendorf)

Refrigerator 4°C (LIEBHERR)

Rinsed water station (Millipore)

Spectrophotometer (Pharmacia Biotech)

Thermocycler PCR(Biometra®)

Thermostat shaker (Heidolph Unimax 1010)

UV-trans illuminator (Vilber Lourmat)

Vortex(Hiedolph)

Water bath (GFL®)

Scalpel blade (AESCULAB® No.11)

Bottle for stock solution (NALGENE® , CRYOWARE^{TM})

Sterilization filter (Millex®-GS 0.22 μM)

Parafilm (NESCOfilm)

Appendix 2. List of chemicals

Growth media component for plant

- MS basal salts with B5 vitamins (Duchefa)
- B5 medium (Duchefa)
- Plant Agar (Duchefa)
- D(+) saccharose (Roth)
- MES(2-[N-morpholino] ethane sulfonic acid) (Biomol)

Plant Hormones

Hormone	Molecular weight	Company	Solvent	Stock solution
2,4-D	221.6	Duchefa	KOH	10 mM
Kin	215.2	Duchefa	KOH	10 mM
NAA	186.2	Duchefa	KOH	10 mM
BAP	225.3	Duchefa	KOH	10 mM
TDZ	220.2	Duchefa	KOH	10 mM
Acetosyringone	196.2	Roth	DMSO	10 mM

Antibiotics and selection agents

Substances	Molecular weight	Company	Stock	Solvent
Sulbactam	233.24	Pfitzer	100 mg/ml	Pure H_2O
Kanamycin	582.60	Duchefa	50 mg/ml	Pure H_2O
Ticarcillin	428.39	Duchefa	100 mg/ml	Pure H_2O
Phosphinotricin (PPT)	198.16	Roth	600 mg/l	Pure H_2O

Appendix 3. List of buffers and solutions

Solutions and buffers for GUS assay (Jefferson et al., 1987)

- **100mM Sodium Phosphate, pH 7.0**
    - 57.7 ml 1M Na_2HPO_4 and 42.3 ml 1M NaH_2PO_4
- **Gus staining solution**
    - 100 mM Sodium phosphate, pH 7.0
    - 1 mM EDTA,pH 8.0
    - 5 mM Potassium ferrocynide
    - 1 % Triton X-100
    - Add X-Gluc at a concentration of 0.5 mg/ml (Dissolve X-Gluc in DMSO)

Buffer for Quick method of genomic DNA isolation (Edwards et al., 1991):

- 200 mM Tris-HCl pH 7.5
- 250 mM NaCl
- 25 mM EDTA
- 0.5 % SDS

Buffers and solutions for CTAB based DNA isolation (Doyle and Doyle, 1990)

- CTAB buffer:
    - 3% CTAB
    - 1.4 M NaCl
    - 0.2% ß-Mercaptoethanol
    - 20 mM EDTA
    - 100 mM Tris-HCl, pH 8.0
    - 0.5 % PVP-40 Polyvinyl Pyrolidone
- 24:1 CI-Mix: 24 part Chloroform and 1 part Isoamylalcohol
- Wash buffer (WB): 76 % Ethanol and 10 mM Ammonium acetate

- EDTA (0.5 M):
    - 18.612 g in 70 ml H_2O
    - Adjust pH 8.0 with NaOH pellets
    - Bring volume to 100 ml
- TE-buffer: 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA

Buffers and solutions for gel-electrophoresis

- 6x DNA loading Dye (MBI Fermentas/Thermo Scientific):
    - 40 mM Tris-HCl, pH 7.6
    - 0.03% Bromophenol blue
    - 0.03% Xylene cyanol FF
    - 60% Glycerol
    - 60mM EDTA
- 50 X TAE buffer (1000 ml):
    - 242 g Tris-base
    - 57.1 ml Glacial Acetatic acid
    - 100 ml 0.5 M EDTA pH 8.0
- 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA) from 50x stock
    - 100 ml 50 X TAE
    - 4900 ml double distilled water
- Redsafe™ Nucleic Acid Staining solution (20, 000x) (iNtRON Biotechnology)

GoTap® Flexi DNA polymerase (Promega) component

- GoTaq® DNA polymerase
- 5X Green GoTaq® Flexi Buffer
- 5X colorless GoTaq® Flexi Buffer
- Magnesium Chloride solution, 25mM

DNA markers

- Gene Ruler™ 100 bp plus DNA ladder (MBI Fermentas/Thermo Scientific)

Appendix 4. Summary of the molecular and functional characterization of Cry1Ac transgenic pea plants

No.	Transgenic line	Progeny level	Germination/grafting	PCR			LP
				HMG	Cry1Ac	Bar499	
1	A1-9-1	T2	+	+	-	-	-
2	A2/D12	T0	+	+	+	+	+
3	A2/D12-1-1	T2	+	+	+	+	+
4	A2/D12-1-1-1-1	T4	+	+	+	+	+
5	A2/D12-1-2	T2	+	+	+	+	+
6	A2/D12-1-2-1	T3	+	+	-	+	+
7	A2/D12-1-3	T2	+	+	+	+	+
8	A2/D12-1-4	T2	+	+	+	+	+
9	A2R-2-1	T2	+	+	+	+	-
10	A3	T0	+	+	+	+	+
11	A9R-1	T2	+	+	-	-	-
12	B	T0	+	+	+	+	+
13	B-1-1	T2	-				
14	B1-1-1	T2	+	+	+	+	-
15	B1-1-2	T2	+	+	+	+	+
16	B1-1-3	T2	+	+	+	+	±
17	B1-2-1	T2	+	+	+	+	±
18	B2	T0	+	+	+	+	+
19	B20R-2-1	T2	+	+	-	+	±
20	B2R	T0	+	+	+	+	+
21	B3	T0	+	+	+	+	+
22	B3-1-11-4-1	T4	+	+	+	+	+
23	B3-1-4-1	T3	+	+	+	+	+
24	B3-1-4-2	T3	+	+	+	+	+
25	B3-1-4-3	T3	+	+	+	+	+
26	B3-1-4-4	T3	+	+	+	-	+
27	B3-1-4-5	T3	+	+	+	-	+
28	B3-1-4-6	T3	+	+	+	+	+
29	B3-3-1	T2	+	+	+	+	+
30	B3-3-1-1	T3	+	+	-	-	-
31	B3-3-1-2	T3	+	+	+	+	+
32	B3-3-1-3	T3	+	+	-	-	-
33	B3-3-1-4	T3	+	+	+	+	+
34	B3-3-1-5	T3	+	+	+	+	-
35	B3-3-1-6	T3	-				
36	B3-3-2-1	T3	+	+	+	+	+

Appendix 4. Continuation

37	B3-3-2-2	T3	+	+	+	+	+
38	B3-3-2-3	T3	-				
39	B3-3-2-4	T3	+	+	+	+	+
40	BR	T0	+	+	+	+	+
41	BR*	T0	+	+	+	+	+
42	BR-3-1	T2	+	+	+	+	+
43	BR-3-1-1	T3	+	+	-	-	-
44	BR-3-2	T2	+	+	-	-	-
45	BR-5-1	T2	+	+	+	+	+
46	BR-5-1-1-1	T4	+	+	+	+	+
47	BR-5-1-4-1	T4	+	+	+	+	+
48	BR-5-2	T2	+	+	+	+	+
49	C1-1-1	T3	-				
50	C1-1-2	T3	+	+	+	+	-
51	C1-1-2-1	T3	+	+	+	+	-
52	C1-2-3-3-1	T4	+	-	-	-	-
53	C1-2-3-3-2	T4	+	+	+	-	+
54	C1-2-3-3-3	T4	+	+	+	+	+
55	C1-2-3-3-3-1-1	T6	+	+	+	+	+
56	C1-2-3-3-3-2-1	T6	+	+	+	+	+
57	C1-2-3-3-3-3-1	T6	+	+	+	+	+
58	C1-2-3-3-4	T4	+	+	+	+	+
59	C1-2-3-3-5	T4	+	+	+	+	+
60	C1-2-3-6-2	T5	+	+	+	+	+
61	C1-2-6-13-1	T5	+	+	+	+	+
62	C1-2-6-13-1-2-1	T6	+	+	+	+	+
63	C1-2-6-13-1-3-6-1	T7	+	+	+	+	+
64	C1-2-6-13-2	T5	+	+	+	+	+
65	C1-2-6-13-3	T5	+	+	+	+	+
66	C1-2-6-13-4	T5	+	+	+	+	
67	C1-2-6-13-5	T5	+	+	+	+	+
68	C1-2-6-15-1	T5	+	+	+	+	+
69	C-5-1	T2	+	+	+	+	-
70	C5-1-1	T3	+	+	+	+	-
71	C5-2-1	T3	+	+	+	+	-
72	C5-2-1-1	T4	-				
73	C5-2-2	T3	+	+	+	+	-
74	C5-2-2-1	T4	+	+	+	+	±
75	C5-3-1	T3	+	+	+	+	-
76	C5-4-1	T3	+	+	+	+	-
77	C7	T0	-	+	+	+	+

Appendix 4. Continuation

78	C7-1	T2	+	+	+	+	-
79	C7-1-3-1	T3	+	+	+	+	-
80	C7-1-4-1	T3	+	+	+	+	-
81	C7-1-4-2	T3	+	+	+	+	-
82	C7-1-4-3	T3	+	+	+	+	-
83	C8R-1-1	T2	+	+	+	+	-
84	D	T0	+	+	+	+	+
85	D1	T0	+	+	+	+	+
86	D20	T0	+	+	+	+	+
87	D21R	T0	+	+	+	+	+
88	D2R	T0	+	+	+	+	+
89	D2R-1	T2	+	+	+	+	+
90	D2R-2-12-1	T3	+	+	+	+	+
91	D2R-2-8-1	T3	-				
92	D2R-2-9-5	T3	+	+	+	+	+
93	D40	T0	+	+	+	+	+
94	D4R	T0	+	+	+	+	+
95	DA	T0	+	+	+	+	+
96	DA-2-1	T2	+	+	+	+	+
97	DA-2-1-1-1	T4	+	+	+	+	+
98	DA-2-1-2-1	T4	+	+	-	-	-
99	DA-2-1-4-1	T4	+	+	+	+	+
100	DA-2-2	T2	+	+	+	-	-
101	DA-2-3	T2	+	+	+	+	+
102	DA-2-3-6-1	T4	+	+	+	+	+
103	DA-2-3-9-1	T4	+	+	+	+	+
104	DA-2-4	T2	+	+	+	+	+
105	DA-2-5	T2	+	+	+	+	+
106	DA-2-5-1	T3	+	+	+	+	+
107	DAnne	T0	+	+	+	+	+
108	Ddiff	T0	+	+	+	+	+
109	DqR	T0	+	+	+	+	+
110	DqR-8-1	T3	+	+	+	+	+
111	DqR-8-2	T3	+	+	+	+	+
112	DqR-8-3	T3	+	+	+	+	+
113	DqR-8-4	T3	+	+	+	+	+
114	DqR-8-5	T3	+	+	+	+	
115	DqR-8-6	T3	+	+	-	-	-
116	DqR-8-7	T3	+	+	+	+	+
117	DqR-8-7-1-1	T4	+	+	+	+	+

Appendix 4. Continuation

118	DqR-8-7-2-1	T4	+	+	+	+	+
119	DqR-8-8	T3	+	+	+	+	+
120	DR*	T0	+	+	+	+	+
121	DR11-1	T2	-				
122	DR-2-1	T2	-				
123	DR21R-2-1	T2	+	+	-	-	-
124	DR21R-2-1-1	T3	-				
125	DR21R-2-2	T2	+	+	-	-	-
126	DR21R-2-3	T2	+	+	-	-	-
127	DT	T0	+	+	+	+	+
128	E	T0	+	+	+	+	+
129	E1	T0	+	+	+	+	+
130	E1 5(a)-2-1	T2	+	+	+	+	+
131	E 5(4)-2-1	T2	-				
132	E8	T0	+	+	+	+	+
133	E8R	T0	+	+	+	+	+
134	ER	T0	+	+	+	+	+
135	G	T0	+	+	+	+	+
136	G*A	T0	+	+	+	+	+
137	G*A-	T2	+	+	-	-	-
138	G*A-1-1	T2	+	+	+	+	+
139	G3	T0	+	+	+	+	+
140	G4	T0	+	+	+	+	+
141	G51-1-1-1	T3	-				
142	G51-1-5-1	T3	+	+	+	+	+
143	G51-1-10-1	T3	+	+	+	+	+
144	G51-1-11-1	T3	+	+	+	+	+
145	G51-2-1	T2	-				
146	G51-2-1-1	T3	+	+	-	+	+
147	G51-2-2-1	T3	+	+	+	+	
148	G51-2-2-1-1	T4	+	+	-	-	±
149	G51-2-2-2	T3	-				
150	G51-2-2-3	T3	+	+	+	+	+
151	G51-2-2-3-1-1	T5	+	+	+	+	+
152	G51-2-2-4	T3	+	+	-	-	-
153	G51-2-5-1	T3	+	+	+	+	+
154	GB	T0	+	+	+	+	+
155	GBR	T0	+	+	+	+	+
156	GqR'/GTR'	T0	+	+	+	+	+
157	Control plants(-)		+	+	-	-	-
160	Positive Control (+)		+	-	+	+	NA

Appendix 5. RNA quality and concentration

No.	Line	Progeny	A230 (A)	A260 (A)	A2680 (A)	A320 (A)	A260/A280	A260/A230	µg/µl
1	A2/12-1-3	T2	0.134	0.305	0.145	0.001	2.111	2.286	0.608
2	A2R-2-1	T2	0.150	0.356	0.168	0.000	2.119	2.373	0.712
3	B1-2-1	T2	0.273	0.335	0.156	0.000	2.147	1.227	0.670
4	D2R-1	T2	0.127	0.289	0.138	0.001	2.102	2.286	0.576
5	E1 5(a)-2-1	T2	0.127	0.281	0.131	-0.001	2.136	2.203	0.564
6	G*A-1-1	T2	0.191	0.289	0.141	0.001	2.057	1.516	0.576
7	B3-1-11-4-1	T4	0.125	0.299	0.142	-0.001	2.098	2.381	0.600
8	C5-1-1	T3	0.186	0.450	0.213	0.000	2.113	2.419	0.900
9	C5-3-1	T3	0.164	0.391	0.183	-0.001	2.130	2.376	0.784
10	C7-1-3-1	T3	0.135	0.316	0.148	0.000	2.135	2.341	0.632
11	C7-1-4-1	T3	0.135	0.316	0.177	0.000	2.085	2.365	0.738
12	D2R-2-12-1	T3	0.178	0.439	0.203	-0.003	2.146	2.442	0.884
13	G51-1-11-1	T3	0.158	0.379	0.178	-0.001	2.123	2.390	0.760
14	G51-1-5-1	T3	0.151	0.380	0.177	-0.002	2.134	2.497	0.764
15	A2/D12-1-1-1-1	T4	0.167	0.414	0.194	-0.002	2.122	2.462	0.832
16	BR-5-1-4-1	T4	0.188	0.429	0.204	0.002	2.114	2.296	0.854
17	C5-2-2-1	T4	0.170	0.406	0.191	0.000	2.126	2.388	0.812
18	DA-2-3-6-1	T4	0.144	0.359	0.165	-0.003	2.155	2.463	0.724
19	DqR-8-7-2-1	T4	0.134	0.328	0.155	0.000	2.116	2.448	0.656
20	C1-2-6-15-1	T5	0.120	0.316	0.144	-0.006	2.147	2.556	0.644
21	G51-2-2-3-1-1	T5	0.153	0.400	0.182	-0.005	2.166	2.563	0.810
22	C1-2-3-3-3-2-1	T6	0.138	0.362	0.165	-0.005	2.159	2.566	0.734
23	C1-2-6-13-1-3-6-1	T7	0.143	0.370	0.170	-0.005	2.143	2.534	0.750
24	Control (-C) plant		0.116	0.198	0.093	0.000	2.129	1.707	0.396

Appendix 6. Transgenic plants grouping by expression folds of *cry1Ac* gene

Grouping scale:

High level: > 1 folds normalized expression
Moderate: 0.5-1 folds normalized expression
Low: 0.1- 0.499 folds normalized expression
Very low: < 0.1 folds normalized expression

Line	Expression	Expression SD	Group by expression level	No. of larvae died(out of five inoculated)	Estimated feeding damage(5)
Control	0.000	0.000		1 (0.78)	60 (40)
A2/D12-1-1-1-1	4.718	0.711	high	5	5
BR-5-1-4-1	2.853	1.213	high	5	5
C1-2-6-13-1	2.615	1.249	high	5	10
G51-1-5-1	2.356	0.247	high	5	5
D2R-2-12-1	2.197	0.318	high	5	5
DA-2-1	2.153	1.213	high	5	5
B1-2-1	1.840	0.247	high	3	25
DqR-8-7-2-1	1.679	0.283	high	5	5
G51-1-11-1	1.472	0.441	high	5	5
DA-2-3-6-1	1.460	0.541	high	5	5
C1-2-6-15-1	1.366	0.216	high	3	25
C1-2-6-13-2-3-6-1	1.000	0.396	moderate	2	20
G51-2-2-3-1-1	0.924	0.190	moderate	5	5
C1-2-6-13-2	0.617	0.420	moderate	2	15
C1-2-3-3-3-2-1	0.600	0.107	moderate	5	5
DqR-8-2	0.438	0.063	low	4	5
B3-1-11-4-1	0.410	0.106	low	4	10
DA-2-4	0.392	0.170	low	3	5
DA-2-3	0.335	0.101	low	4	5
B3-3-2-1	0.327	0.187	low	3	15
G*A-1-1	0.155	0.036	low	5	5
D2R-1	0.140	0.053	low	5	5
DqR-8-4	0.090	0.077	very low	5	5
E15(a)-2-1	0.085	0.017	very low	5	5
C1-2-6-13-3	0.082	0.028	very low	3	5
A2R-2-1	0.033	0.011	very low	1	35
B3-3-1-5	0.018	0.004	very low	2	50
B3-1-4-2	0.004	0.002	very low	2	15
A2/D12-1-3	0.002	0.000	very low	5	5
DA-2-2	0.000	0.000	very low	4	10

Appendix 7. Segregation data

Generation	Line	Leaf paint assay using Herbicide					PCR for Cry1Ac				
		Resistant	Susceptible	Total	Test ratio	X^{2*}	Positive	Negative	Total	Test ratio	X^{2*}
T1	DA	5	0	5	3:1	0.00	5	0	5	3:1	0.00
	DqR	2	3	5	3: 1	1.47	2	3	5	3: 1	1.47
	DN	0	5	5	0 :1	0.00	5	0	5	1 : 0	0.00
	A9R	0	5	5	0 :1	0.00	0	5	5	0 :1	0.00
	B3	11	0	14**	3 : 0	0.00	11	0	14	3 : 0	0.00
	BR	6	0	6	1 : 0	0.00	6	0	6	1 : 0	0.00
	C1	2	4	6	3: 1	2.31	6	0	6	3: 1	0.00
	C4	2	5	7	3: 1	3.16	6	1	7	3: 1	0.17
T2	DqR	11	3	19**	3: 1	0.01	10	3	13	3: 1	0.01
	BR-1	1	0	2**	3: 1	0.50	1	0	2	3: 1	0.50
	B3-1	11	0	11	1 : 0	0.00	11	0	11	1 : 0	0.00
	B3-3	5	1	6	3: 1	0.09	5	1	6	3: 1	0.09
	C1-2-1	1	0	1	3:1	0.50	1	0	1	3: 1	0.50
	BR-3	1	1	2	3: 1	0.50	1	1	2	3: 1	0.50
	BR-5	2	0	2	1 : 0	0.00	2	0	2	1 : 0	0.00
	DA-2	4	1	5	3: 1	0.02	5	0	5	1 : 0	0.00
	BR-3	1	1	2	3 : 1	0.50	1	1	2	3 : 1	0.50
	BR-5	2	0	2	3 : 1	0.50	2	0	2	3 : 1	0.50
T3	DqR-8	6	1	7	3: 1	0.17	7	1	8	3: 1	0.25
	B3-1-4	6	0	6	1: 0	0.00	6	0	6	1: 0	0.00
	B3-3-1	2	3	5	3: 1	1.47	5	0	5	1: 0	0.00
	B3-3-2	3	0	3	1: 0	0.00	3	0	3	1: 0	0.00
	BR-1-1	8	1	9	3: 1	0.33	8	1	9	3: 1	0.33
	C1-2-1-2	8	1	9	3: 1	0.33	8	1	9	3: 1	0.33
T4	BR-1-1-3	6	7	13	3: 1	5.77	6	7	13	3: 1	5.77
	C1-2-1-2-1	7	5	12	3: 1	0.59	7	5	12	3: 1	0.59
	C1-2-1-2-2	4	0	4	1 : 0	0.00	4	0	4	1 : 0	0.00
	C1-2-1-2-3	11	0	11	1 : 0	0.00	11	0	11	1 : 0	0.00
	C1-2-1-2-4	14	0	14	1 : 0	0.00	14	0	14	1 : 0	0.00
	C1-2-1-2-5	5	1	6	3: 1	0.09	5	1	6	3: 1	0.09
	C1-2-1-2-6	15	0	15	1 : 0	0.00	15	0	15	1 : 0	0.00
	C1-2-1-2-7	12	3	15	3: 1	0.06	12	3	15	3: 1	0.06
	C1-2-1-2-8	9	0	9	1 : 0	0.00	9	0	9	1 : 0	0.00
T5	C1-2-1-2-3-3	4	0	4	1: 0	0.44	4	0	4	1: 0	0.000
	C1-2-1-2-6-13	5	0	5	1: 0	0.53	5	0	5	1: 0	0.000

Appendix 8. Effect of explant orientation during co-cultivation on the pattern of *GUS* gene expression

Explants	Age (day) of explants	Treatment	Explant Orientation	Number of Explants with			Transformation efficiency (%)	Explants with strong GUS expression		
				Blue spot	No Blue spot	Total		Part of explants	No. Explants	Explants (%)
CN0(=DE)	0	GUS	Vertical upright	26	0	26	100.00	Epicotyl region	20	76.92
		Control	Vertical upright	0	15	15	0.00			
		GUS	Vertical inverted	39	3	42	92.86	Hypocotyl region	31	73.81
		Control	Vertical inverted	0	12	12	0.00			
		GUS	Horizontal	30	0	30	100.00			
		Control	Horizontal	0	9	9	0.00			
CN1	1	GUS	Vertical upright	27	0	27	100.00	Epicotyl region	20	74.07
		Control	Vertical upright	0	8	8	0.00			
		GUS	Vertical inverted	30	2	32	93.75	Hypocotyl region	21	65.63
		Control	Vertical inverted	0	8	8	0.00			
		GUS	Horizontal	25	0	25	100.00			
		Control	Horizontal	0	8	8	0.00			
CN2	2	GUS	Vertical upright	10	1	11	90.91	Epicotyl region	5	45.45
		GUS	Vertical inverted	10	0	10	100.00	Hypocotyl region	8	80.00
		GUS	Horizontal	7	2	9	77.78			
		Control	--	0	9	9	0.00			
CN3	3	GUS	Vertical upright	24	1	25	96.00	Epicotyl region	16	64.00
		Control	Vertical upright	0	9	9	0.00			
		GUS	Vertical inverted	20	3	23	86.96	Hypocotyl region	14	60.87
		Control	Vertical inverted	0	10	10	0.00			
		GUS	Horizontal	19	2	21	90.48			
		Control	Horizontal	0	10	10	0.00			

Acknowledgement

This work would not have been in its today's form without the support, encouragement, contribution and involvement of many people for whom I feel very happy and grateful.

First of all, I am deeply grateful to my supervisor Prof. Dr. Hans-Jorg Jacobsen for all his valuable guidance, advices, comments, suggestions, encouragements, kindness and supports during my study time. I also like to extend special thanks for his invaluable contributions, constructive criticism and reviewing of my thesis which not only contributed to the quality of the thesis itself but also helped me a lot in my professional development. His everyday tips have always kept me encouraged and motivated to learn and do more day-by-day and have a vision on my career. I am also greatly thankful to him to allow me to work in his well equipped laboratory. He has trusted and had faith on me for which I also like to extend my heartfelt thanks to him.

I would like to thank Prof. Dr. Edgar Maiß for being a Korreferent to my work and for all his suggestions, advises and comments. His fruitful discussion and guidance are also deeply acknowledged.

I am deeply grateful to Dr. Fathi Hassan for his guidance, advices, comments and supports. His invaluable guidance, constructive criticism and suggestions have been very crucial to bring my study to its current form. Without his day-to-day guidance and support, it would be unthinkable to go through the long process.

I would like to thank Dr. Jürgen Langewald (BASF Plant Science, Limburgerhof, Germany) for kindly providing larvae of tobacco budworm (*Heliothis virescens*) used for the feeding test. I am also very thankful to him for his important suggestions on the insect bioassay experiments.

Special thanks Kenyan friends (Daniel, James, Josephine, Jacinter and others) who helped in getting cowpea seeds used in this study. I would like to thank Mr. Timo Michel for his valuable supports and helps during the insect bioassay experiments.

I would like to express my deepest gratitude and thanks to all plant biotechnology department (Institute of plant genetics) staffs Dr. Thomas Reinard, Dr. Maren Wichmann, Nicole Giesemann, Beate Meyer, Sandra Keil and Julia Schröter. Without their supports and helps it

would have been very difficult to integrate into the working environment and use most of the high valued facilities in the laboratory.

I shared office with Linda Kirchner and Dr. Sibel Day for whom I would like to extend special thanks for their discussion, kindness and friendship that contributed to the success of my works in one or another. I also like thanks current/former students (Robin Pfeil, Peter Stoffels, Tim Wembeuer, Andeas Buchbender, Linda Baranek, Songly You, Hi Ha, Daniel Herlihy, Hamza Mohamad and others) of the department for their supports, kindness and friendship during my stay in the department.

I like to thanks my friend Dr. Diro T. Ayan for his all round supports and kindness during my study time. Special thanks Dr. Yetenayet B. Tola, Dr. Tesfaye Belami, Chala Kinati and Umar Dido for their encouragement and motivation. I also like to thank Mr. Dheressa Kenea and his family for their support and helps.

I like to extend special thanks to my wife, Galane Fayisa, for her unreserved supports, understanding and encouragements, and to look after our daughters, Sena and Nunahi, during my study time. Her presence has been a lot to me and helped me to concentrate more on pursuing my academic career. Thank you a lot again and again! I also like to thank my daughters for their patience and understanding. They also deserve my special thanks for their special love which I could not able to express in a million of words and pay back in a million of years.

I have had a lot supports from my brothers during my study time for which they deserve my special thanks all the time. I also like to extent special thanks my brother Abera for his inspiration, encouragements and motivations (particularly since I started high school) every time I talked to him. I like to express my appreciation and thanks to my father Teressa Negawo and my mother Askela Chemeda who have supported and brought me to the road from where I came to the place where I am today. It is their vision and determination which leads to my current professional development. Thank you very much for your invaluable contribution to my success.

I am very grateful to GIZ for the financial support.

Finally, I would like to thank God for his presence and blessings. He gave me all the patience and endurance to be who I am today. Thank you God!!

Curriculum vitae

Personal data

Full Name:	Alemayehu Teressa Negawo
Birth Date:	December 31, 1981
Birth Place and Country:	Showa, Oromia, Ethiopia
Nationality:	Ethiopian
Sex:	Male
Marital Status:	Married
Language:	English, Afan Oromo, Amharic and German (Basic)

Educational background:

Oct. 2012-now: PhD student at Plant Biotechnology Department, Institute of Plant Genetics**,** Gottfried Wilhelm Leibniz Universität Hannover, Germany.

Oct. 2010 – Sept. 2012: M.Sc. in International Horticulture (Major in Plant Biotechnology). Gottfried Wilhelm Leibniz Universität Hannover, Germany. **(Graduated with 'Summa cum laude')**

Thesis: Transgenic insect resistance in pea (*Pisum sativum* L.)

Oct. 2006- Oct. 2007: DIPLÔME D'INITIATION À LA RECHERCHE SCIENTIFIQUE, University of Tours, France **(Graduated with 'Tres Bien')**.

Thesis: Genetic Diversity of *Coffea arabica* L. Collections using Microsatellite (SSRs) Markers

Sept. 1999 - July 2003: B.Sc. in Horticulture, Jimma University, Ethiopia

Sept 1995- July 1999: Ethiopians school leaving certificate, Ethiopia

Publication

Alemayehu Teressa Negawo, Mitra Aftabi, Hans-Jörg Jacobsen, Illima Altosaar and Fathi Hassan. 2013. Insect resistant transgenic pea expressing cry1Ac gene product from Bacillus thuringiensis. *Biological Control*, 67, 293-300.

Alemayehu Teressa, Dominique Crouzillat, Vincent Petiard and Pier Brouhan. 2010. Genetic diversity of Arabica coffee (*Coffea arabica* L.) collections. *EJAST 1(1): 63-79.*

Zerihun Abebe, Ayelign Mengesha, Alemayehu Teressa and Wondyifraw Tefera. 2009. Efficient *in vitro* multiplication protocol for *Vanilla planifolia* using nodal explants in Ethiopia. African Journal of Biotechnology Vol. 8 (24), pp. 6817-682.

Zerihun Abebe, Ayelign Mengesha, Alemayehu Teressa, and Wondyifraw Tefera, 2009. *In vitro* multiplication, somaclonal variation and field performance of pineapple *(Ananas comosus* (L)) cv Smooth Cayanne in Ethiopia, *19th Biological Society of Ethiopia Annual conference*. Addis Ababa, Ethiopia.

Ramos RA, Wondyifraw T, Martinez F, Gonzalez ME, Endale G, Alemayehu T and Zerihun A. 2009. Plant regeneration through somatic embryogenesis in three Ethiopian *Coffea arabica* Lin. Hybrids. *Biotecnologia Vegetal vol. 9, No. 1: 19-26.*

Wondyifraw T., Ramos. A, Felipe. M, Alemayehu T. and Zerihun A. 2007. Tissue Culture Multiplication Techniques for Coffee. In: Adugna G, Bellachew B, Shimbre T, Taye E, and Kufa T (Eds). Proceedings of National Workshop Four Decades of Coffee research and Development in Ethiopia. Pp. 106-119. Addis Ababa, Ethiopia.

Conference contributions

Alemayehu Teressa Negawo, Fathi Hassan and Hans-Jörg Jacobsen. 2014. Regeneration and *Agrobacterium*-mediated transformation of cowpea. Oral presentation at Tropentag, September 17-19, 2014. Prague, Czech Republic.

Alemayehu Teressa Negawo, Fathi Hassan and Hans-Jörg Jacobsen. 2014. Development of Insect and Fungi Resistant Transgenic pea (*Pisum Sativum* L.). Poster at IFLRC VI & ICLGG VII. July 7-11, 2014. Saskatoon, Saskatchewan, Canada.

Alemayehu Teressa Negawo, Hans-Jörg Jacobsen and Fathi Hassan. 2012. Development of Insect Resistant Transgenic Pea (*Pisum sativum* L.): Molecular and Functional Characterization of Putative Transgenic Pea Plants. Poster at Tropentag 2012, Göttingen, Germany.

Alemayehu T.N, Petiard V, Broun P and Crouzillat D. 2010. Molecular Genetic Diversity of Arabica coffee (Coffea arabice L.) using SSR markers. Poster at the 23rd International Conference on Coffee Science. 3-8 October 2010. Bali, Indonesia.

Crouzillat D, Lefebvre F, Michel1 R, Priyono P, Alemayehu T, Feinan W, Laurence B, Mawardi S, Wahyudi T, Charles L, Tanksley S, Vincent P, and Pierre B. 2008. Use of DNA markers (SSRs & SNPs) for the assessment of the genetic diversity and the genome mapping among Coffea species. The 5th Solanaceae Genome Workshop. Cologne, Germany.

Zerihun Abebe, Wondyifraw Tefera, Ayelign Mengesha, Alemayehu Teressa, and Fillipe M. 2008. I*n vitro* multiplication of pineapple (*Ananas comosus* (L)) cv Smooth Cayanne and Cardamom (*Ellettaria cardamomum*) in Ethiopia, *2nd Biennnial conference of Ethiopian Horticultural society.* Addis Ababa. pp: 9-18.

Biological Control 67 (2013) 293–300

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

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

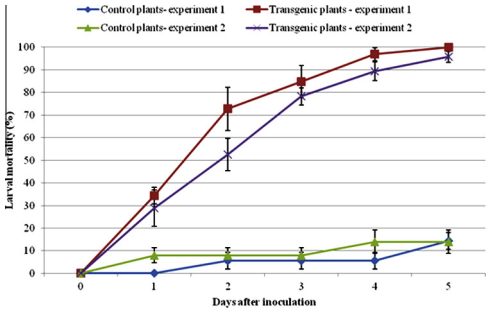
Alemayehu Teressa Negawo [a], Mitra Aftabi [a], Hans-Jörg Jacobsen [a], Illimar Altosaar [b], Fathi S. Hassan [a,*]

[a] Institute for Plant Genetics, Section of Plant Biotechnology, Gottfried Wilhelm Leibniz Universität Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany
[b] Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

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



ARTICLE INFO

Article history:
Received 10 March 2013
Accepted 20 September 2013
Available online 29 September 2013

Keywords:
Pea
Transgenic plant
Cry1Ac protein
Bt toxin
Insect resistance
Agrobacterium

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.

* Corresponding author. Address: Institute of Plant Genetics, Section of Plant Biotechnology, Leibniz University Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany. Fax: +49 511 7624088.
E-mail address: hassan@lgm.uni-hannover.de (F.S. Hassan).

1049-9644/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biocontrol.2013.09.016

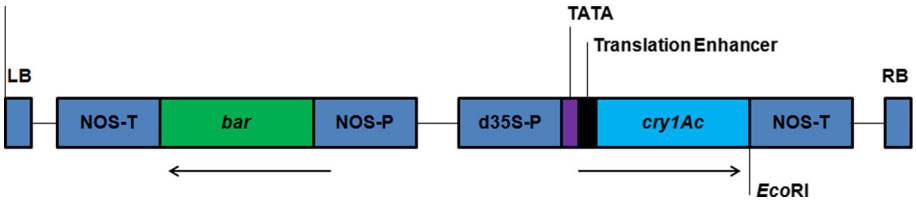


Fig. 1. The physical map of the transformation vector pGII35S-Cry1Ac. d35S-P, double 35S promoter from CaMV; cry1Ac, codon optimized insect resistant gene from *Bacillus thuringiensis* (Sardana et al., 1996; Cheng et al., 1998); NOS-P and NOS-T, *Agrobacterium* nopaline synthase promoter and terminator, 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

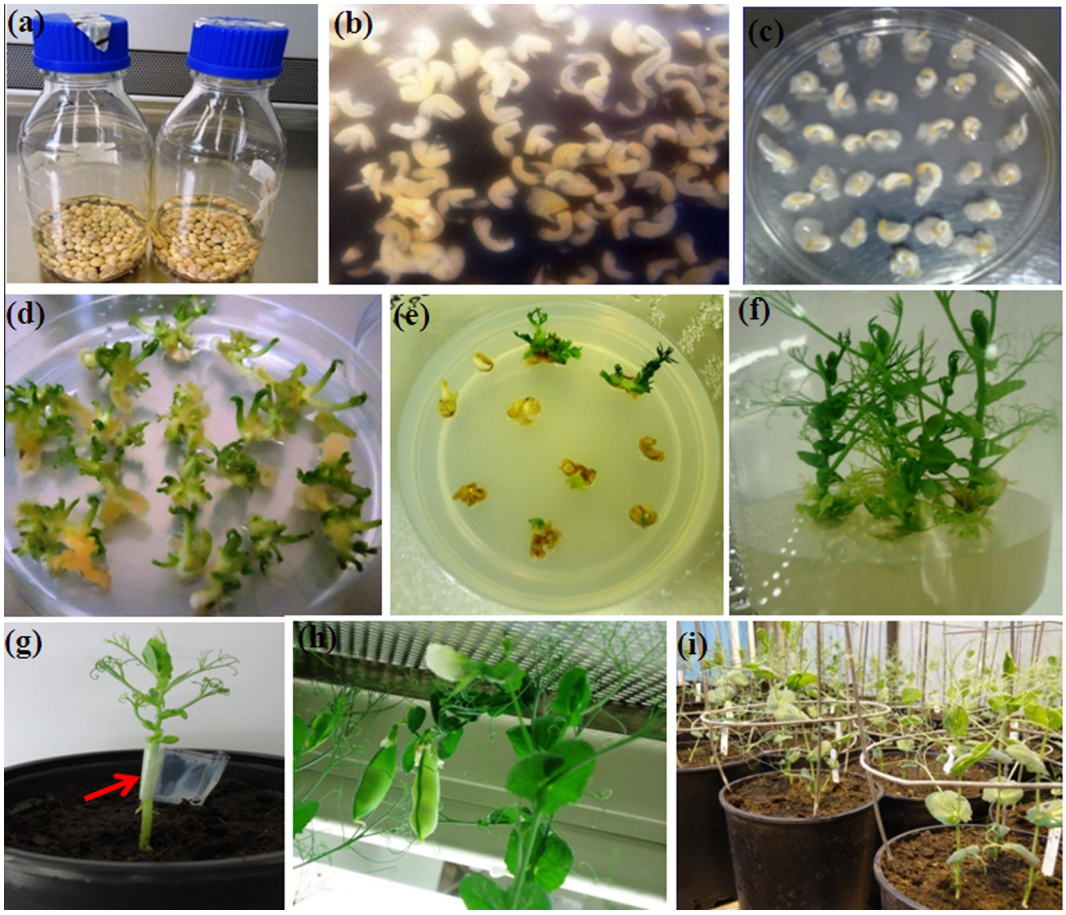


Fig. 2. An overview of transgenic pea development. (a) Seed surface sterilization and overnight soaking in sterile distilled water (Days 1–2). (b) Explants (embryo slices) preparation and inoculation with *Agrobacterium* suspension harboring transformation vector (Days 2). (c) Co-cultivation of explants for 3–4 days under dark condition at 22 ± 2 °C (Days 2–6). (d) Multiple shoot induction from co-cultivated explants on MST medium for 10 days under 16/8 h light/dark at 22 ± 2 °C (Days 6–16). (e) Selective regeneration of shoots on P2 medium supplemented with 2.5–10 mg/l PPT at 3 weeks interval (Days 16–90). (f) Putative transgenic shoots on P2 medium supplemented with 10 mg/l PPT ready for micro-grafting. (g) Recovery of putative transgenic shoots through micro-grafting on seedling rootstock (The arrow indicates the graft union tied with tape). During the first two weeks, it was necessary to maintain high relative humidity around the grafted shoots in order to facilitate graft-union healing. (h) Successfully grafted putative transgenic shoots with flower and pods (arrows). (i) Greenhouse grown subsequent generations.

Table 1
List of primers, their annealing temperature and the expected PCR product.

Target gene	Primers	Tm[a] (°C)	PCR product
Cry1Ac	Forward: 5′-GTTCAGGAGAGAATTGACCC-3′ Reverse: 5′-CTTCACTGCAGGGATTTGAG-3′	56	750 bp
Bar	Forward: 5′-CTACCATGAGCCCAGAACGACG-3′ Reverse: 5′-CTGCCAGAAACCCACGTCATGCCAGTTC-3′	62	499 bp
hmg-I/Y	Forward: 5′-ATGGCAACAAGAGAGGTTAA-3′ Reverse: 5′-TGGTGCATTAGGATCCTTAG-3	56	570/350 bp[b]

[a] Tm: Annealing temperature.
[b] 570 bp for genomic DNA and 350 for cDNA.

gel (w/v) and then transferred to a positively charged nylon membrane (Roche, Germany) as described by Sambrook et al. (1989). The membrane was hybridized with DIG-labeled Cry1Ac probe using PCR-DIG Mix (Roche, Germany) according to the manufacturer's instructions. The blots were exposed to X-ray film for 1 h.

2.4. RT-PCR expression analysis of cry1Ac gene

Total RNA was isolated from young leaves using NucleoSpin® RNA plant kit (Macherely-Nagel) according to the manufacturer's instructions. Five micrograms of the total RNA was used for cDNA synthesis using RevertAid™ H Minus cDNA synthesis kit (MBI Fermentas, St Leon-Rot, Germany). The cDNA was used as a template for PCR amplification of the transgene specific fragment.

2.5. Detection of Cry1Ac protein

The leaves of transgenic plants were collected and used to check the presence of Cry1Ac protein using an immunostrip assay (Bt-Cry1Ab/1Ac ImmunoStrip® test, Agdia Inc., USA) following the manufacturer's instructions. The leaf sample (80–100 mg per plant) was ground using liquid nitrogen and crude protein was extracted using 1.5 ml 1× sample extraction buffer 4 (SEB4,

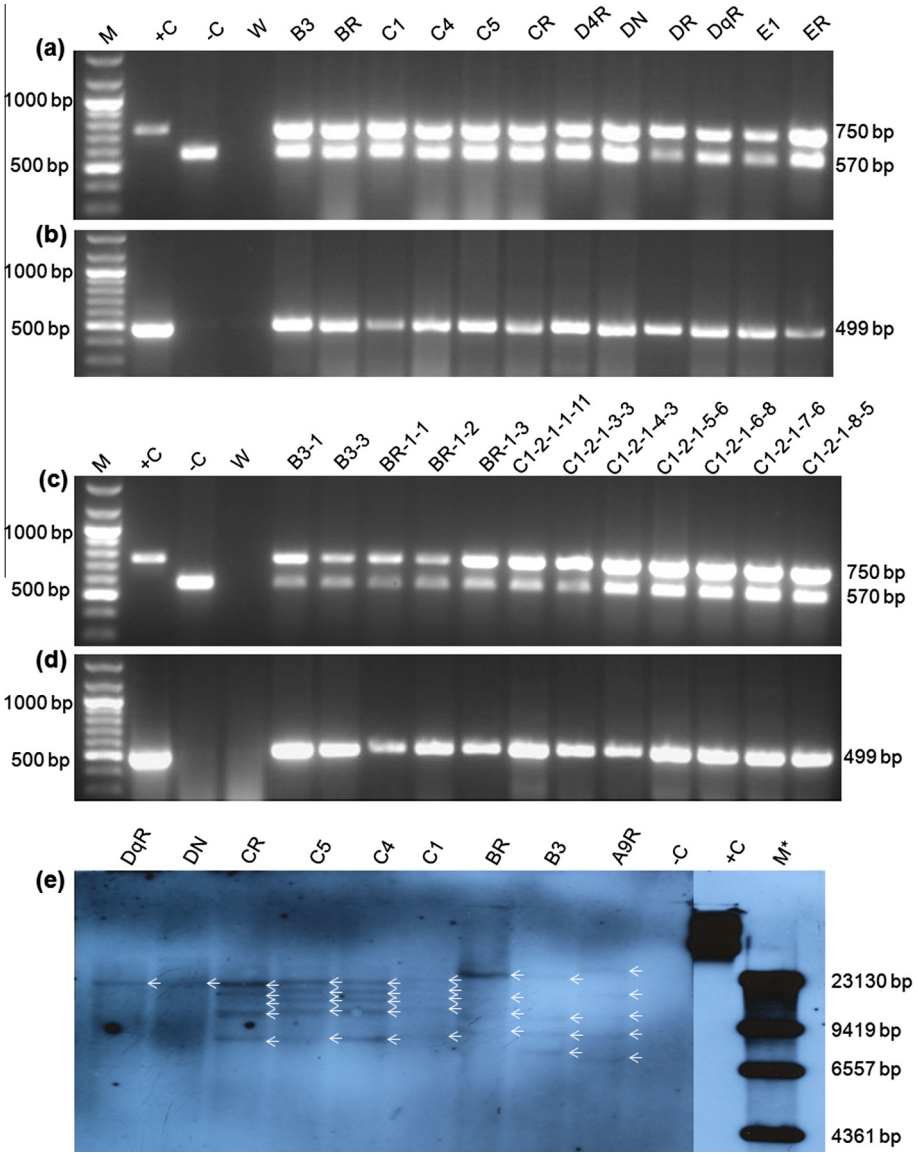


Fig. 3. Molecular confirmation of the stable integration and inheritance of the T-DNA region into the genome of pea plants. (a) Multiplex PCR detection of *cry1Ac* (product size of 750 bp) and *HMG* (product size of 570 bp) genes in putative transgenic plants (T0). (b) PCR detection of *bar* gene (product size of 499 bp) in putative transgenic plants. (c) Multiplex PCR detection of *cry1Ac* and *HMG* genes in the subsequent generations (T1 plants: B3-1 and B3-3; T3 plants: BR-1-1, BR-1-2 and BR-1-3; T4 plants: C1-2-1-1-11, C1-2-1-3-3, C1-2-1-4-3, C1-2-1-5-6, C1-2-1-6-8, C1-2-1-7-6 and C1-2-1-8-5) of transgenic plants. (d) PCR detection of *bar* gene in the subsequent generations of transgenic plants. (e) Southern blot analysis of transgenic plants (T0) using DIG labeled *cry1Ac* probe. +C: plasmid DNA (pGII35S-cry1Ac) as a positive control, −C: genomic DNA of non-transgenic plant as a negative control, W: water control, M: GeneRuler™ 100 bp plus DNA ladder (MBI Fermentas, St Leon-Rot, Germany) and M*: DIG-labeled DNA molecular weight marker II (Roche, Germany).

Agdia Inc., USA). Then, 500 μl of the crude extract was transferred to a new microcentrifuge tube and a Bt-Cry1Ab/1Ac Immunostrip® was inserted into the tube. Signal development on the strip was noted after 30 min for the presence of Cry1Ac protein.

2.6. Leaf paint functional assay

Leaf paint functional characterization of progenies from transgenic clones was conducted according to Schroeder et al. (1993) with modification following Richter et al. (2006). 600 mg/l herbicide solution BASTA® (Aventis GmbH, Frankfurt, Germany) was used. One pair of leaves was selected on the actively growing progenies of transgenic and non-transgenic control plants. From the selected pair of leaves, one of the leaves was marked as a control while the prepared herbicide solution was applied on the upper surface of the other leaf using a small brush. Then, the relative herbicide tolerance of the plants was evaluated visually one week after application.

2.7. Insect bioassay

To test the efficiency of transgenic plants' resistance against insect pests, insect bioassays were conducted twice using the larvae of tobacco budworm (*Heliothis virescens*) kindly provided by Dr. Jürgen Langewald (BASF Limburgerhof, Germany). T4 and T5 generation of transgenic plants and non-transgenic control plants were grown under contained condition and used for feeding experiments. In the first experiment, seven each transgenic and non-transgenic plants were used, while for the second experiment, 10 plants each were used. Five larvae were inoculated on each plant.

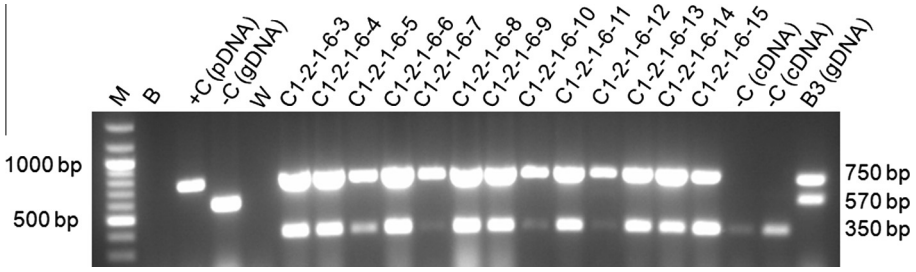


Fig. 4. RT-PCR expression analysis of *cry1Ac* (750 bp) and *HMG* (570 bp for gDNA and 350 bp for cDNA) genes in T4 transgenic plants.+C (pDNA): plasmid (pGII35S-cry1Ac) DNA as a positive control, −C (gDNA) and −C (cDNA): genomic DNA and cDNA, respectively, from non-transgenic plant as a negative control, B3 (gDNA): genomic DNA from clone B3 (T0 plant) as a control, B: blank, W: water control and M: GeneRuler™ 100 bp plus DNA ladder.

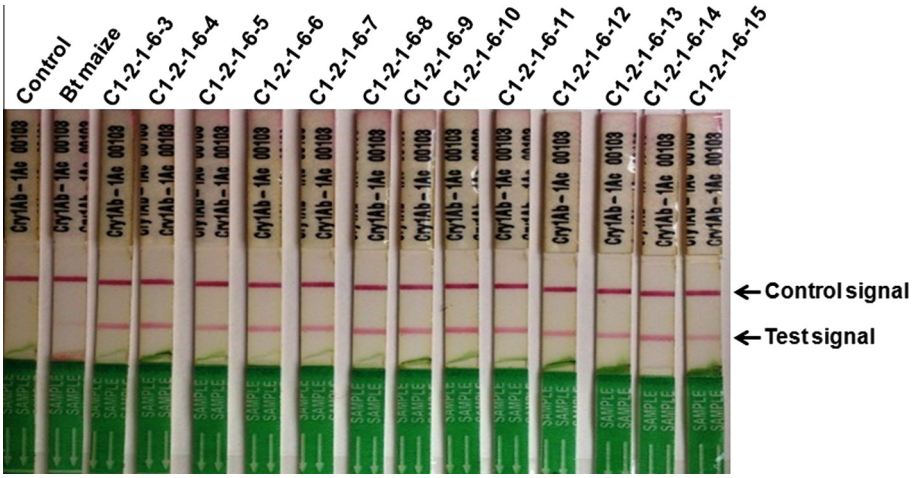


Fig. 5. Immunostrip detection of Cry1Ac protein in the leaves of some T4 generation plants. Control: crude protein extract from non-transgenic pea plant, Bt maize: crude protein extract from seeds of Bt maize and C1-2-1-6-N (where N ranges from 3–15): crude protein extract from leaves of transgenic pea plants. The control signals proof the function of the test as well as the non-transgenic control plant, while the transgenic lines indicated the positive plants.

Then, larval survival and feeding damage were evaluated daily for one week post-inoculation.

3. Results

3.1. Genomic integration of the T-DNA region

Putative transgenic pea plants expressing *cry1Ac* gene for insect resistance and *bar* gene as a plant selectable marker gene were developed through *Agrobacterium* transformation. Selective regeneration and maintenance of the developed putative transgenic shoots were done on medium supplemented with PPT. Based on the described procedure (Section 2.1), a minimum 7–9 months were required to get the putative transgenic shoots ready for micro-grafting. *In vitro* putative transgenic shoots from more than 65 clones out of 2500 explants were recovered by micro-grafting and analyzed using PCR and Southern blotting. PCR analysis was done for all recovered putative transgenic shoots while Southern blotting was done for few selected lines.

The results of PCR analysis using *cry1Ac* and *bar* gene specific primers (Fig. 3a and b) indicated the genomic integration of the T-DNA region and thereby the transgenic nature of the regenerated *in vitro* plants. Further PCR analysis of the subsequent generations (T1–T4) indicated the stable inheritance of the introduced transgenes to the next generations (Fig. 3c and d). The result of Southern blot analysis using DIG labeled non-radioactive cry1Ac probe showed a single copy for two clones (DqR and DN) and five copies for seven clones (A9R, B3, BR, C1, C4, C5 and CR) (Fig. 3e).

3.2. Expression analysis

Transcriptional level analysis using RT-PCR indicated the expression of the *cry1Ac* gene in the developed transgenic plants. Using the prepared cDNA as a template for PCR analysis, the expected PCR fragment for the introduced *cry1Ac* gene was amplified in the transgenic plants while the equivalent PCR fragment was absent in the control plants (Fig. 4).

3.3. Detection of Cry1Ac protein

The immunostrip assay specific to Cry1Ac/Cry1Ab protein indicated the accumulation of the novel protein in the transgenic plants (Fig. 5). The control and test signals were observed in the transgenic lines while only the control signal was observed in the case of non-transgenic control plant.

3.4. Leaf paint functional characterization of progenies from transgenic plants

The result of leaf paint assay showed a clearly observable difference between non-transgenic control plants and progenies from transgenic plants (Fig. 6). The leaf of the control plants was susceptible to the herbicide application. However, in the segregating progenies of transgenic plants, both susceptible and tolerant leaves were observed.

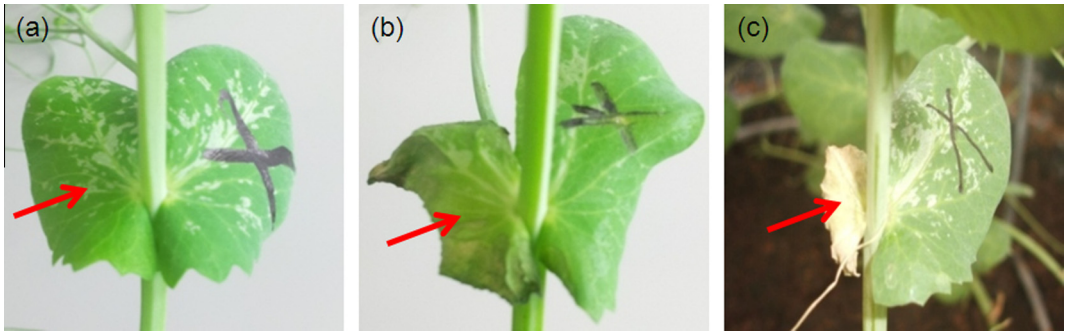


Fig. 6. Leaf paint assay of progenies from cry1Ac transgenic pea plants. (a) herbicide resistant transgenic plant showing no effect, (b) herbicide susceptible transgenic plant and (c) herbicide susceptible non-transgenic control plant. The arrows indicate the herbicide solution (BASTA®, 600 mg/l) treated leaves and the marked leaves indicates the control leaves. The effect of the herbicide was monitored after one week.

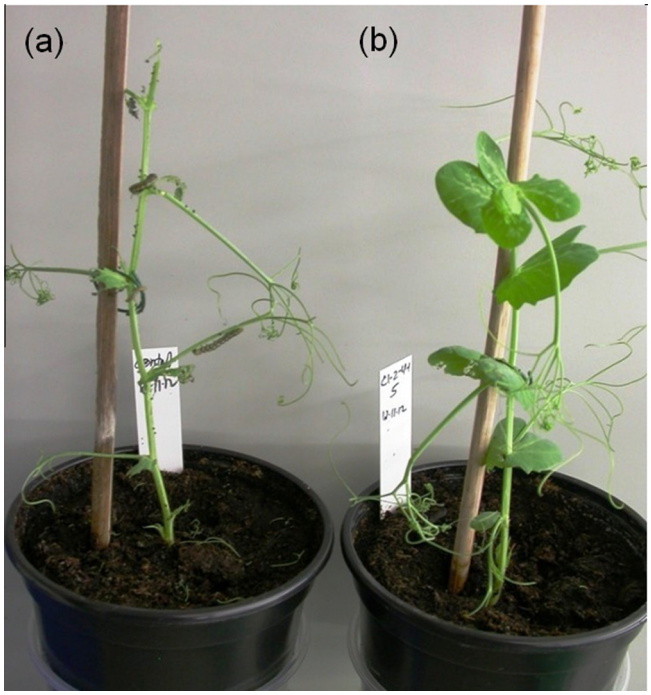


Fig. 7. Insect bioassay on cry1Ac transgenic pea plants with the larvae of tobacco budworm (*Heliothis virescens*. The larvae were obtained from BASF Company) after 5–7 days after inoculation. (a) Non-transformed control pea plants inoculated with five larvae resulted in heavy damage and normal growth of the larvae. (b) Transgenic pea plants with negligible feeding damage and no survival of the inoculated larvae.

3.5. Insect bioassay

In order to evaluate the level of insect resistance in the transgenic pea plants, insect feeding tests were conducted using one of the target pests, tobacco budworm. The result showed a clear difference between transgenic and control plants in term of damage and larval mortality. The observed feeding damage was negligible on transgenic plants while the control plants were totally damaged by the inoculated larvae (Fig. 7). Larval survival was also highly impaired on the transgenic plants where most of the larvae (73–85% in the first experiment and 53–78% in the second experiment) died within 2–3 days after inoculation on the transgenic plants which was not the case on the non-transgenic control plants (Fig. 8). 5–7 days after inoculation, none of the larva survived on transgenic plants whereas about 85% of the larvae survived on the non-transgenic control plants.

4. Discussion

Insect pests are the major cause of yield losses of grain legumes throughout the world. About three decades have passed since transgenic approaches were applied to improve crops wherever conventional methods had no options, like in the case of insect resistance. Nowadays insect resistant transgenic crops are under production on increased acreage throughout the world (James, 2011). However, the use of transgenic approaches has been limited to some major crops while there is also an urgent need for the application of this technology in most of our crops. Grain legumes such as pea, despite a multipurpose socio-economically important crop and constrained significantly by insect pests, are an example of crops that have less benefited from the application of genetic transformation.

In this study, we developed and analyzed transgenic pea plants expressing a *cry1Ac* gene conferring insect resistance and *bar* gene as a plant selectable marker. The stable genomic integration, inheritance and expression of the introduced transgenes were confirmed using PCR, Southern blot and RT-PCR analyses.

Besides molecular analyses at the DNA and RNA level, it is also necessary to determine whether the introduced transgene is translated to the intended Cry1Ac protein product. Accordingly, the accumulation of the novel Cry1Ac protein in the developed transgenic plants was demonstrated by an immunostrip assay specific to Cry1Ac/Cry1Ab proteins. A similar technique was used to detect the presence of Cry1Ab protein in transgenic potato (Hagh et al., 2009) and three Cry proteins (Cry1Ac, Cry2Ab and Cry1F) in transgenic cotton genotypes (Ali et al., 2012).

Leaf paint functional assay was conducted on progenies obtained from PCR confirmed transgenic clones to evaluate the *bar* gene expression and activity. Similar assays have been used to characterize progenies of different transgenic pea plants (Hassan et al., 2009; Richter et al., 2006). They reported that transgenic plants that inherited the *bar* gene are resistant to herbicide application. The result of the current study is also in line with previous reports. Accordingly, both herbicide susceptible and resistant plants were observed in the progenies from transgenic plants. This suggested segregation since the putative transgenic plants (T0) were not homozygous for the transgenes (*bar* and *cry1Ac* genes). The original transgenic (T0) plants obtained from transformation experiments were hemizygous for the introduced transgenes (Zale, 2008). In the subsequent generations (T1 onward), a mixture of zygosity level (such as homozygous for the presence of transgene, hemizygous for the transgene and homozygous for the absence of transgene) are expected (Zale, 2008; Sridevi et al., 2006). At advanced generations such as T2, T3 and T4, it is possible to identify

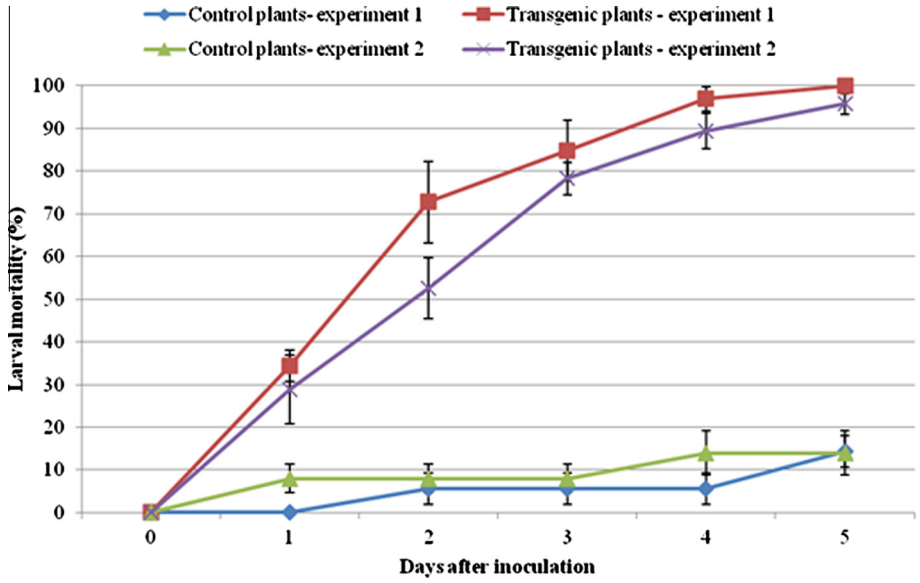


Fig. 8. Effect of transgenic pea plants on larval mortality. Five to seven days after larval inoculation, total larval mortality was recorded on cry1Ac transgenic pea plants compared to about 15% larval mortality on non-transgenic control plants.

and recover homozygous lines where all subsequent progenies could show resistance to the herbicide application.

The result of insect bioassays demonstrated the resistance of the developed transgenic lines to one of the target insect pests. Total larval mortality and negligible feeding damage was observed on the transgenic plants compared to 15% larval mortality and heavy feeding damage on the control plants. Similar results have been reported on transgenic crops expressing different cry proteins. Transgenic sorghum plants expressing a synthetic *cry1Ac* gene showed 40% larval mortality of spotted stem borer (Girijashankar et al., 2005). Total mortality of tobacco budworm and tobacco hornworm larvae and negligible insect damage has been observed on transgenic tobacco expressing cry1Ac protein (Gulbitti-Onarici et al., 2009). Similarly, up to 100% larval mortality of yellow stem borer was reported on transgenic rice expressing cry1Ac protein (Cheng et al., 1998).

In general, the development of insect resistant transgenic pea expressing cry1Ac protein has been confirmed by molecular and functional analyses and would be useful in the future of pea improvement programs such as transgene stacking. It would also be interesting to see how the soil bacteria such as nodule forming Rhizobia would be affected in the rhizosphere of the developed transgenic plants. Furthermore, conducting field trials would also be necessary to evaluate the performance of the developed transgenic lines under natural growing conditions.

Acknowledgment

The authors would like to thank Dr. Jürgen Langewald (BASF Plant Science, Limburgerhof, Germany) for kindly providing larvae of tobacco budworm used in the feeding experiment. First author would like to thank The German Academic Exchange Service (DAAD) for scholarship support.

References

Ali, S., Shah, S.H., Ali, G.M., Iqbal, A., Asad, M.A.U., Zafar, Y., 2012. Bt Cry toxin expression profile in selected Pakistani cotton genotypes. Curr. Sci. 102, 1632–1636.

Biddle, A.J., Cattlin, N.D., 2001. Pests and Diseases of Peas and Beans – A Colour Handbook. Manson Publishing Ltd., London.

Carozzi, N., Koziel, M., 1997. Advances in insect control: the role of transgenic plants. Taylor & Francis, London.

Cheng, X.Y., Sardana, R., Kaplan, H., Altosaar, I., 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. U.S.A. 95 (6), 2767–2772. http://dx.doi.org/10.1073/pnas.95.6.2767.

Clement, S.L., Hardie, D.C., Elberson, L.R., 2002. Variation among accessions of *Pisum fulvum* for resistance to pea weevil. Crop Sci. 42, 2167–2173.

Clement, S.L., McPhee, K.E., Elberson, L.R., Evans, M.A., 2009. Pea weevil, *Bruchus pisorum* L. (Coleoptera: Bruchidae), resistance in *Pisum sativum* x *Pisum fulvum* interspecific crosses. Plant Breeding 128, 478–485.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D.H., 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62 (3), 807–813.

Doyle, J.J., Doyle, J.L., 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus 12, 13–15.

Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D.E., Gresshoff, P.M., 2010. Molecular analysis of legume nodule development and autoregulation. J. Integr. Plant Biol. 52 (1), 61–76.

Gatehouse, J.A., 2008. Biotechnological prospects for engineering insect-resistant plants. Plant Physiol. 146, 881–887.

Girijashankar, V., Sharma, H.C., Sharma, K.K., Swathisree, V., Prasad, L.S., Bhat, B.V., Royer, M., Secundo, B.S., Narasu, M.L., Altosaar, I., Seetharama, N., 2005. Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). Plant Cell Rep. 24, 513–522.

Graham, P.H., Vance, C.P., 2003. Legumes: importance and constraints to greater use. Plant Physiol. 131, 872–877.

Gulbitti-Onarici, S., Zaidi, M.A., Taga, I., Ozcan, S., Altosaar, I., 2009. Expression of Cry1Ac in transgenic tobacco plants under the control of a wound-inducible promoter (AoPR1) isolated from *Asparagus officinalis* to control *Heliothis virescens* and *Manduca sexta*. Mol. Biotechnol. 42, 341–349.

Gupta, R., Webster, C.I., Gray, J.C., 1997. The single-copy gene encoding high-mobility-group protein HMG-I/Y from pea contains a single intron and is expressed in all organs. Plant Mol. Biol. 35, 987–992.

Hagh, Z.G., Rahnama, H., Panahandeh, J., Rouz, B.B.K., Jafari, K.M.A., Mahna, N., 2009. Green-tissue-specific, C-4-PEPC-promoter-driven expression of Cry1Ab makes transgenic potato plants resistant to tuber moth (*Phthorimaea operculella*, Zeller). Plant Cell Rep. 28, 1869–1879.

Hassan, F., Meens, J., Jacobsen, H.J., Kiesecker, H., 2009. A family 19 chitinase (Chit30) from *Streptomyces olivaceoviridis* ATCC 11238 expressed in transgenic pea affects the development of *T. harzianum in vitro*. J. Biotechnol. 143, 302–308, doi: http://dx.doi.org/10.1016/j.jbiotec.2009.08.011.

Hofte, H., Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242–255.

James, C., 2011. Global Status of Commercialized Biotech/GM Crops: BRIEF 43. ISAAA, Metro Manila, Philippines.

Keneni, G., Bekele, E., Getu, E., Imtiaz, M., Damte, T., Mulatu, B., Dagne, K., 2011. Breeding food legumes for resistance to storage insect pests: potential and limitations. Sustainability 3, 1399–1415. http://dx.doi.org/10.3390/su3091399.

Korth, K.L., 2008. Genes and traits of interest for trangenic plants. In: Stewart, C.N. (Ed.), Plant Biotechnology and Genetics: Principles, Techniques, and Applications. John Wiley & Sons Inc., Hoboken, NJ, USA.

Lee, R.Y., Reiner, D., Dekan, G., Moore, A.E., Higgins, T.J.V., Epstein, M.M., 2013. Genetically Modified α-Amylase Inhibitor Peas Are Not Specifically Allergenic in Mice. PLoS One 8 (1), e52972. http://dx.doi.org/10.1371/journal.pone.0052972.

Legowski, T.J., Gould, H.J., 1960. Losses of dry harvesting peas due to pea moth in East Anglia and the economics of control measures. Plant. Pathol. 9, 119–126.

Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K., Thompson, C.J., 1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205 (1), 42–50.

Oelke, E.A., Oplinger, E.S., Hanson, C.V., Davis, D.W., Putnam, D.H., Fuller, E.I., Rosen, C.J., 1991. Dry field pea. Center for New Crops and Plant Products, Purdue University.

Perlak, F.J., Deaton, R.W., Armstrong, T.A., Fuchs, R.L., Sims, S.R., Greenplate, J.T., Fischhoff, D.A., 1990. Insect resistant cotton plants. Biotechnology (NY) 8 (10), 939–943.

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D., Fischhoff, D.A., 1993. Genetically improved potatoes: protection from damage by Colorado potato beetles. Plant Mol. Biol. 22 (2), 313–321.

Pickardt, T., Saalbach, I., Waddell, D., Meixner, M., Muntz, K., Schieder, O., 1995. Seed specific expression of the 2S albumin gene from Brazil Nut (*Bertholletia excelsa*) in transgenic *Vicia narbonensis*. Mol. Breeding 1, 295–301.

Prescott, V.E., Campbell, P.M., Moore, A., Mattes, J., Rothenberg, M.E., Foster, P.S., Higgins, T.J.V., Hogan, S.P., 2005. Transgenic expression of bean α-amylase inhibitor in peas results in altered structure and immunogenicity. J. Agric. Food Chem. 53 (23), 9023–9030.

Richter, A., de Kathen, A., de Lorenzo, G., Briviba, K., Hain, R., Ramsay, G., Jacobsen, H.J., Kiesecker, H., 2006. Transgenic peas (*Pisum sativum*) expressing polygalacturonase inhibiting protein from raspberry (*Rubus idaeus*) and stilbene synthase from grape (*Vitis vinifera*). Plant Cell Rep. 25 (11), 1166–1173.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sardana, R., Dukiandjiev, S., Giband, M., Cheng, X.Y., Cowan, K., Sauder, C., Altosaar, I., 1996. Construction and rapid testing of synthetic and modified toxin gene sequences CryIA (b&c) by expression in maize endosperm culture. Plant Cell Rep. 15 (9), 677–681.

Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D., Higgins, T.J.V., 1993. Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). Plant Physiol. 101, 751–757.

Schroeder, H.E., Gollasch, S., Moore, A., Tabe, L.M., Craig, S., Hardie, D.C., Chrispeels, M.J., Spencer, D., Higgins, T.J.V., 1995. Bean [alpha]-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). Plant Physiol. 107, 1233–1239, doi: http://dx.doi.org/10.1104/pp.107.4.1233.

Schuler, T.H., Poppy, G.M., Kerry, B.R., Denholm, I., 1998. Insect-resistant transgenic plants. Trends Biotechnol. 16, 168–175.

Sridevi, G., Parameswari, C., Rajamuni, P., Veluthambi, K., 2006. Identification of hemizygous and homozygous transgenic rice plants in T_1 generation by DNA blot analysis. Plant Biotechnol. 23, 531–534, doi: http://dx.doi.org/10.5511/plantbiotechnology.23.531.

Williams, L., Schotzko, D.J., Okeeffe, L.E., 1995. Pea leaf weevil herbivory on pea seedlings: effects on growth response and yield. Entomol. Exp. Appl. 76, 255–269.

Zaidi, M., El Bilali, J., Koziol, A., Ward, T., Styles, G., Greenham, T., Faiella, W., Son, H., Wan, S., Taga, I., Altosaar, I., 2012. Gene technology in agriculture, environment and biopharming: beyond Bt-rice and building better breeding budgets for crops. J. Plant Biochem. Biotechnol. 21 (1), 2–9.

Zale, J., 2008. Transgenic plant analysis. In: Stewart, C.N. (Ed.), Plant Biotechnology and Genetics: Principles, Techniques, and Applications. John Wiley & Sons Inc., pp. 275–289.