



# Genetic engineering studies on insect resistant tobacco plants expressing *Bacillus thuringiensis* insecticidal proteins

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(Degree)

博士 (学術)

(Date of Degree)

1999-03-31

(Date of Publication)

2013-10-23

(Resource Type)

doctoral thesis

(Report Number)

甲1899

(JaLCD0I)

<https://doi.org/10.11501/3156300>

(URL)

<https://hdl.handle.net/20.500.14094/D1001899>

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博士論文

**GENETIC ENGINEERING STUDIES ON INSECT  
RESISTANT TOBACCO PLANTS EXPRESSING *BACILLUS*  
*THURINGIENSIS* INSECTICIDAL PROTEINS**

平成 11 年 1 月

神戸大学大学院自然科学研究科

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**BT 殺虫蛋白質を発現した昆虫耐性タバコ植物に  
関する遺伝子工学的研究**

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*THURINGIENSIS* INSECTICIDAL PROTEINS**

**A dissertation for partial fulfillment of a Doctoral Degree at the  
Graduate School of Science and Technology,  
Kobe University**

**January, 1999**

**Nikolai Kirilov Christov**



**“There are no such things  
as applied sciences, only  
applications of science”**

*Louis Pasteur (1822–95).*



# TABLE OF CONTENTS

|                                                                                                                                                              |           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| ABBREVIATIONS .....                                                                                                                                          | III       |
| LIST OF FIGURES .....                                                                                                                                        | IV        |
| LIST OF TABLES .....                                                                                                                                         | V         |
| <b>CHAPTER I GENERAL INTRODUCTION.....</b>                                                                                                                   | <b>1</b>  |
| 1.1 BACILLUS THURINGIENSIS AND ITS INSECTICIDAL CRYSTAL PROTEINS .....                                                                                       | 3         |
| 1.2 EXPRESSION OF INSECTICIDAL PROTEIN GENES IN TRANSGENIC PLANTS .....                                                                                      | 8         |
| 1.3 PURPOSE OF THE STUDY .....                                                                                                                               | 15        |
| <b>CHAPTER II EXPRESSION OF GENETICALLY ENGINEERED <u>CRY 1AB</u> GENES FROM <u>BACILLUS THURINGIENSIS</u> SSP. <u>AIZAWAI</u> IN TOBACCO PLANTS.....</b>    | <b>19</b> |
| 2.1 INTRODUCTION .....                                                                                                                                       | 21        |
| 2.2 MATERIALS AND METHODS .....                                                                                                                              | 22        |
| 2.2.1 Construction of recombinant plasmids .....                                                                                                             | 22        |
| 2.2.2 Tobacco transformation.....                                                                                                                            | 24        |
| 2.2.3 Southern blot.....                                                                                                                                     | 24        |
| 2.2.4 Genomic PCR.....                                                                                                                                       | 24        |
| 2.2.5 NPT II activity gel assay .....                                                                                                                        | 25        |
| 2.2.6 RT-PCR experiments.....                                                                                                                                | 25        |
| 2.2.7 Bioassay with <i>Spodoptera litura</i> larvae.....                                                                                                     | 26        |
| 2.3 RESULTS AND DISCUSSION.....                                                                                                                              | 26        |
| 2.3.1 Recombinant plasmid constructs .....                                                                                                                   | 26        |
| 2.3.2 Southern blot analyses .....                                                                                                                           | 27        |
| 2.3.3 Insecticidal activity .....                                                                                                                            | 29        |
| 2.3.4 RT-PCR detection of spliced <i>cry 1Ab</i> transcripts. ....                                                                                           | 33        |
| <b>CHAPTER III GREEN TISSUE SPECIFIC EXPRESSION OF GENETICALLY ENGINEERED <u>CRY 1C</u> GENES FROM <u>BACILLUS THURINGIENSIS</u> IN TOBACCO PLANTS .....</b> | <b>37</b> |
| 3.1 INTRODUCTION.....                                                                                                                                        | 39        |
| 3.2 MATERIALS AND METHODS .....                                                                                                                              | 41        |
| 3.2.1 Design of the synthetic <i>cry 1C</i> gene coding sequence .....                                                                                       | 41        |
| 3.2.2 Construction of recombinant plasmids .....                                                                                                             | 45        |
| 3.2.3 Tobacco transformation.....                                                                                                                            | 47        |
| 3.2.4 NPT II ELISA .....                                                                                                                                     | 47        |
| 3.2.5 Genomic PCR.....                                                                                                                                       | 48        |
| 3.2.6 RT-PCR experiments.....                                                                                                                                | 48        |
| 3.2.7 Northern blot.....                                                                                                                                     | 49        |
| 3.2.8 Protein extraction and Western blot .....                                                                                                              | 49        |
| 3.2.9 Chlorotoluron Selection of transgenic plants .....                                                                                                     | 50        |
| 3.2.10 Bioassay with <i>S. litura</i> Larvae .....                                                                                                           | 51        |
| 3.2.11 Production of dihaploids and germination tests.....                                                                                                   | 51        |
| 3.3 RESULTS.....                                                                                                                                             | 52        |
| 3.3.1 Construction of the synthetic <i>cry 1C</i> gene .....                                                                                                 | 52        |

|                                                                                                                                            |           |
|--------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 3.3.2 Expression of the truncated wild type <i>cry 1C</i> gene in haploid tobacco plants .....                                             | 54        |
| 3.3.3 Coexpression of both truncated wild type <i>cry 1C</i> and <i>rat CYP1A1/yeast P450 oxidoreductase</i> genes in tobacco plants ..... | 58        |
| 3.3.4 Expression of the truncated synthetic <i>cry 1C</i> gene in tobacco plants .....                                                     | 64        |
| 3.4 DISCUSSION .....                                                                                                                       | 69        |
| <b>CHAPTER IV CONCLUDING REMARKS.....</b>                                                                                                  | <b>75</b> |
| SUMMARY .....                                                                                                                              | 83        |
| ACKNOWLEDGMENTS .....                                                                                                                      | 86        |
| REFERENCES.....                                                                                                                            | 87        |
| PUBLICATIONS.....                                                                                                                          | 95        |

## ABBREVIATIONS

|          |                                                           |
|----------|-----------------------------------------------------------|
| BCIP     | 5-bromo-4-chloro-3-indolylphosphate p-toluidine           |
| Bt       | <i>Bacillus thuringiensis</i>                             |
| CTP      | cytidine 5'-triphosphate                                  |
| CaMV 35S | cauliflower mosaic virus 35S promoter                     |
| CYP1A1   | rat P 450 1A1                                             |
| dNTP     | deoxy nucleotide 5'-triphosphate                          |
| ELISA    | enzyme linked immuno sorbent assay                        |
| EDTA     | ethylene diamine tetraacetic acid                         |
| Km       | kanamycin                                                 |
| NBT      | nitro-blue tetrazolium chloride                           |
| NPT II   | neomycin phospho-transferase                              |
| PCR      | polymerase chain reaction                                 |
| PVDF     | polyvinilide difluoride                                   |
| RH       | relative humidity                                         |
| RT-PCR   | Reverse Transcription PCR                                 |
| RuBisCo  | ribulose biphosphate carboxylase oxygenase                |
| rbcS     | ribulose biphosphate carboxylase-oxygenase small subunit  |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TBE      | tris borate EDTA                                          |
| T-DNA    | transfer DNA                                              |
| YR       | yeast NADPH-cytochrome P 450 oxidoreductase               |

## LIST OF FIGURES

- Fig. 2-1. Schematic representation of the recombinant plasmids for the expression of the truncated wild type and mutant *cry 1Ab* genes in tobacco plant.
- Fig. 2-2. Southern blot of BamHI digested total DNA from transgenic tobacco plants expressing the mutant and the wild type truncated *cry 1Ab* genes.
- Fig. 2-3. Insecticidal activity of the wild type (BTW) and a mutant (BTM) *cry 1Ab* expressing tobacco plants towards neonate *Spodoptera litura* larvae.
- Fig. 2-4. In situ NPT II activity of total leaf protein extracts from the transgenic tobacco plants.
- Fig. 2-5. RT-PCR of polyadenylated RNA from the tobacco plants transformed with plasmids pBTTW (BTW) and pBTM (BTM).
- Fig. 3-1. Nucleotide sequence of the reconstructed truncated *cry 1C* gene.
- Fig. 3-2. Schematic representation of the recombinant expression plasmids pRBS1CS and pBSynC1 for the expression of the wild type and synthetic *cry 1C* genes, and the plasmid pCGFN for the coexpression of both the wild type *cry 1C* gene and *rat CYP1A1/yeast P450 oxidoreductase* fusion gene.
- Fig. 3-3. NPT II protein levels and the insecticidal activities of transgenic tobacco plants expressing the wild type *cry 1C* gene under the control of *rbcS* promoter.
- Fig. 3-4. Screening of kanamycin resistant tobacco plants coexpressing wild type *cry 1C* and *rat CYP1A1/yeast P450 oxidoreductase (CYP1A1/YR)* genes.
- Fig. 3-5. Detection of *CYP1A1/YR* and *cry 1C* transcripts in tobacco plants coexpressing wild type *cry 1C* and *CYP1A1/yeast P450 oxidoreductase* genes. M, marker; 1, untransformed control; 2, CGN19; 3, CGN21; 4, CGN22; 5, CGN36; 6, CGN39.
- Fig. 3-6. The content of *rat CYP1A1/yeast P450 oxidoreductase (CYP1A1/YR)* protein and the insecticidal activity in the chlorotoluron resistant haploid tobacco plants coexpressing wild type *cry 1C* and *CYP1A1/YR* genes.
- Fig. 3-7. Expression of the truncated synthetic *cry 1C* gene under the control of *rbcS* promoter in haploid tobacco plants.
- Fig. 3-8. Detection of the *cry 1C* transcripts in tobacco plants expressing the synthetic *cry 1C* gene by northern blot.
- Fig. 3-9. Tissue specific expression of the Cry 1C protein in the transgenic tobacco line RSC23 expressing the synthetic *cry 1C* gene.
- Fig. 3-10. Detached leaf bioassay of transgenic tobacco plants expressing each wild type *cry 1C* gene or synthetic *cry 1C* gene with neonate *Spodoptera litura* larvae.

## LIST OF TABLES

Table 1-1 *Bacillus thuringiensis* insecticidal crystal protein genes and the insecticidal specificity of the encoded proteins.

Table 1-2 Characteristics of synthetic Bt toxin genes expressed in plant cells.

Table 3-1 Codon Usage in a Truncated wild type *cry 1C* Gene Compared to that of 4 Dicotyledonous. RuBisCo Small Subunit Genes.

Table 3-2 Summary of selection of pRBS1CS transformed tobacco plants expressing truncated wt *cry 1C* gene.

Table 3-3 Kanamycin resistance of the T1 progeny of tobacco plants, transformed. with the plasmid pBSynC1.



# **Chapter I**

## **General introduction**



## **1.1 BACILLUS THURINGIENSIS AND ITS INSECTICIDAL CRYSTAL PROTEINS**

Modern agriculture utilizes a number of agrochemicals for control of insect pests, plant pathogens as well as weeds. However, public has much concerns about environmental contamination and human health with relation to the use of toxic chemicals for plant protection. Therefore, alternatives, which are safer to human health and environment, are requested in the world. One practical alternative to chemical pesticides is the development of biopesticides based on bacteria, viruses, and fungi, which are natural pathogens of susceptible pests. Among many of biopesticides developed, the most widely used is *Bacillus thuringiensis* (Bt) insecticides, which has advantages including high specificity, low toxicity to mammals, plants and beneficial insects as well as low persistence in the environment.

*Bacillus thuringiensis* is a gram positive spore forming soil bacterium, which was first discovered in diseased silkworms in Japan [1]. During a late stage of sporulation, this bacterium produces crystal protein inclusions which are highly insecticidal towards certain lepidopteran, coleopteran or dipteran insect species [2]. Although the shape of the protein crystals differs depending on the bacterial species, all of them are called  $\delta$ -endotoxins. In the crystals most of the proteins occur as inactive protoxins ranging in size from 65 kDa to 140 kDa. When ingested by the susceptible insects, the crystalline protoxins are solubilized at the high alkaline pH of the insect midgut and cleaved by gut juice proteases to release mature toxins of approximately 55 to 65 kDa [3, 4]. These active fragments bind to receptors present on epithelial cells of the midgut of the susceptible insects [5] and probably cause formation of pores in the membrane of these cells [6] leading to cell lysis and finally results in death of the susceptible larvae. Recently a cDNA encoding a receptor from tobacco hornworm (*Manduca sexta*) for an insecticidal protein of *B. thuringiensis* was cloned and

characterized [7]. The receptor was a 210-kDa membrane glycoprotein, which specifically binds the Cry IA(b) toxin of *B. thuringiensis subsp. berliner*. The receptor protein also showed sequence similarity to cadherin protein superfamily. The cDNA for a another receptor specific to Cry IA(c) toxins was cloned from *Heliothis virescens* [8]. The receptor was reported to be a 120 kD brush border membrane glycoprotein which shared homology to the aminopeptidase N protein family. These results suggested that the action of Bt toxin at the cell membranes including receptor binding and pore formation was important process for the activity. However, it was demonstrated that a native or hybrid toxins, showing little or no intoxication *in vivo* were very active towards epithelial cells by *in vitro* assays [9], suggesting that factors other than receptor binding may contribute to determination of the insecticidal activity *in vivo*.

The insecticidal protein genes of *B. thuringiensis* were sometimes found on the chromosome, but usually located on plasmids [10]. The first insecticidal protein gene was cloned from *B. thuringiensis kurstaki* HD-1 [11]. Then, a large number of genes have been cloned and sequenced. Hofte and Whiteley [6] have proposed the unifying nomenclature for the insecticidal protein genes, utilizing *cry* as mnemonic, which is now widely accepted (Table. 1-1). The fundamental basis for this classification is specificity as to insect order: *cry I* is specific towards *Lepidoptera*, *cry II* is active towards *Lepidoptera* and *Diptera*, *cry III* towards *Coleoptera*, *cry IV* towards *Diptera* and two new classes *cry V* and *cry VI* which are active towards *Nematodes*. The *cry* gene classes are further divided into subclasses based on the specificity towards certain insect species and sequence similarity (Table. 1-1). For example, Cry I A proteins share greater than 80% amino acid homology between each other, whereas the sequence similarity to the other Cry I protein subclasses is less than 80 %. Although the high sequence homology was shared by the Cry I A protein subclasses, the members of

these subclasses show differences in insecticidal activity and are highly specific towards certain insect species (Table. 1-1), [6].

Recently, due to the increasing number of cloned insecticidal protein genes, the usage of a nomenclature based on the biological properties is becoming impractical. A new nomenclature was proposed by an international comity for the classification of the Bt insecticidal protein genes [12]. The new nomenclature is based solely on the sequence homology of the genes. The mnemonic *cry* was retained, however the writing is simplified by replacement of the Roman numbers with Arabic numbers, removal of the parenthesis, and by adding an additional number to the alphabetic classification. The names of some representative insecticidal protein genes according to the new nomenclature are given in Table 1-1 in the column "New name" and the most recent classification could be found at: [http://epunix.biols.susx.ac.uk/Home/Neil\\_Crickmore/Bt/index.html](http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html). As far as the new nomenclature does not require testing of the insect specificity of each newly cloned insecticidal protein gene, it is likely to replace the existing nomenclature in the near future. Therefore, the most recent nomenclature [12] will be followed in the present thesis paper.

Table 1-1 *Bacillus thuringiensis* insecticidal crystal protein genes and the insecticidal specificity of the encoded proteins

| Gene             | Gene<br>New name <sup>1</sup> | Insecticidal Specificity |                              |
|------------------|-------------------------------|--------------------------|------------------------------|
|                  |                               | Order                    | Species                      |
| <i>cry IA(a)</i> | <i>cry 1Aa</i>                | <i>Lepidoptera</i>       | <i>Bombyx mori</i>           |
| <i>cry IA(b)</i> | <i>cry 1Ab</i>                | <i>Lepidoptera</i>       | <i>Lymantria dispar</i>      |
|                  |                               | <i>Diptera</i>           | <i>Aedes aegyptii</i>        |
| <i>cry IA(c)</i> | <i>cry 1Ac</i>                | <i>Lepidoptera</i>       | <i>Trychoplusia ni</i>       |
|                  |                               |                          | <i>Heliothis virescens</i>   |
|                  |                               |                          | <i>Pieris brassicae</i>      |
| <i>cry IB</i>    | <i>cry 1Ba</i>                | <i>Lepidoptera</i>       | <i>Pieris brassicae</i>      |
| <i>cry IC</i>    | <i>cry 1Ca</i>                | <i>Lepidoptera</i>       | <i>Mamestra brassicae</i>    |
|                  |                               |                          | <i>Spodoptera littoralis</i> |
| <i>cry ID</i>    | <i>cry 1Da</i>                | <i>Lepidoptera</i>       | <i>Manduca sexta</i>         |
| <i>cry IH</i>    | <i>cry 9Ca</i>                | <i>Lepidoptera</i>       | <i>Mamestra brassicae</i>    |
|                  |                               |                          | <i>Spodoptera littoralis</i> |
| <i>cry IIA</i>   | <i>cry 2Aa</i>                | <i>Lepidoptera</i>       | <i>Lymantria dispar</i>      |
|                  |                               | <i>Diptera</i>           | <i>Aedes aegyptii</i>        |
| <i>cry IIB</i>   | <i>cry 2Ab</i>                | <i>Lepidoptera</i>       | <i>Lymantria dispar</i>      |
| <i>cry IIIA</i>  | <i>cry 3Aa</i>                | <i>Coleoptera</i>        | <i>Leptinotarsa</i>          |
|                  |                               |                          | <i>decemlineata</i>          |
| <i>cry IIIB</i>  | <i>cry 3Ba</i>                | <i>Coleoptera</i>        | not given                    |
| <i>cry IVA</i>   | <i>cry 4Aa</i>                | <i>Diptera</i>           | <i>Culex</i>                 |
| <i>cry IVB</i>   | <i>cry 4Ba</i>                | <i>Diptera</i>           | <i>Aedes</i>                 |
| <i>cry IVC</i>   | <i>cry 10Aa</i>               | <i>Diptera</i>           | <i>Aedes</i>                 |
| <i>cry V</i>     | <i>cry 5Aa</i>                | Nematode                 | not given                    |
| <i>cry VI</i>    | <i>cry 6Aa</i>                | Nematode                 | not given                    |

After [6], modified and updated.

<sup>1</sup> Name according to the most recent classification [12]

Comparison of the derived amino acid sequences of insecticidal crystal protein genes revealed two major structural groups: large size proteins with  $M_r$  of 130-140 kDa (*cry 1* series, and *cry 4A* and *cry 4B*); and small size proteins with  $M_r$  of 60-70 kDa crystal proteins (*cry 2* series; *cry 3A*; *cry 10A* and *cry 11A*).

The activation process of large size proteins of 130-140 kDa was studied *in vitro* by trypsin cleavage. Amino acid analysis of the trypsin-cleaved active fragment of the Cry 1Ac insecticidal protein revealed that location of the active fragment was between Ile29 and Lys 623 at the N-terminus of the protoxin [13]. Deletion analysis of the cloned *cry 1Ab* gene of *B. thuringiensis* Berliner 1715 also demonstrated that the amino acid residues between 27 and 607 were required for the full toxicity [14]. Recently, similar deletion studies of the cloned *cry 1Ab* gene showed that the gene product extending from amino acid residues 29 to 607 appeared to be toxic not only to the insect larvae of *Manduca sexta* and *Heliothis virescens* but also to *E. coli* cells [15]. The authors of this study suggested that the N-terminal 28 amino acids render the toxin inactive before removal of these residues by proteases in the insect midgut.

Amino acid sequence alignment of the small size proteins of 60-70 kDa indicated that Cry 2, Cry 3, Cry 10Aa and Cry 11Aa proteins were homologous to the N-terminal half of the Cry 1 proteins and lack the C-terminal half which was not essential for the insecticidal activity.

The 3-dimensional structure of the mature form of coleoptera-specific Cry 3Aa insecticidal protein has been resolved by X-ray crystallography of the crystallized protein [16]. The protein comprised of three structural domains. Domain I contained 7  $\alpha$ -helixes and was involved in pore formation. Domain II, consisting of 3  $\beta$ -sheets mediated receptor recognition and binding. The  $\beta$ -sandwich of domain III was possibly involved in stabilizing the structure and protecting of the mature form from further digestion by midgut proteases. The core of the

domains consisted of the conserved regions among the insecticidal protein sequence blocks, while the variable regions were exposed to the surface. Therefore it was expected that the similar structural organization was shared by the other members of Cry protein family. The latest was confirmed by the resolution of the structure of the *Lepidoptera* specific Cry 1Aa protein [17]. Although, the Cry 1Aa have an insect specificity which differs significantly from that of the Cry 3A the overall domain organization of the two proteins was surprisingly similar.

## **1.2 EXPRESSION OF INSECTICIDAL PROTEIN GENES IN TRANSGENIC PLANTS**

Since the insecticidal proteins of *Bacillus thuringiensis* are not toxic to plants and animals, a new approach for pest management, based on introduction of *B. thuringiensis* insecticidal protein genes into plants, became available right after the first Cry protein gene have been cloned and characterized [11]. The experiments to generate insect resistant transgenic plants expressing insecticidal protein genes of *B. thuringiensis* started in the early 1980s and the first report came out in 1987. Adang et al. [18] attempted to express an intact *cry 1Ac* gene under the control of mannopine synthase (*mas*) promoter in tobacco plants. The expression level of the insecticidal protein was very low in the transgenic plants when detected by ELISA, while northern blots revealed the presence of only 1.7 kb truncated polyadenylated transcript. It was also shown that the *steady-state* level of this truncated transcript was 100-fold lower than RNA levels from neomycin phosphotransferase (*npt ii*) gene.

Barton et al. [19] examined the expression of intact and truncated (coding for the toxic part) *cry 1Aa* genes from *B. thuringiensis kurstaki* HD 1 in transgenic tobacco plants. The transgene constructs contained intact or truncated *cry 1Aa* gene coding sequence with the optimized translation start codon under the control of CaMV 35S promoter and

AMV 5' untranslated leader. The Cry 1Aa protein was detected in the calli obtained after transformation with the intact gene construct, although such calli were necrotic and died. In contrast, the truncated construct did not appear to interfere with the regeneration ability of the transgenic plants. The authors detected the *cry 1Aa* gene specific mRNA of a predicted size as a minor species on northern blot of 17 µg of oligo-dT selected RNA from transgenic plants expressing the truncated gene and showed that most of the hybridized signals were distinctly shorter polyadenylated transcripts. They also detected the transgene protein by western blot at a level of about 10-15 ng/mg soluble protein and showed that the transgenic tobacco were well protected towards tobacco hornworm (*Manduca sexta*) in laboratory experiments.

Transgenic tobacco plants, harboring intact or truncated *cry 1Ab* gene construct under the control of *mas* promoter, have also been generated [20]. Additional constructs containing truncated *cry 1Ab/npt ii* fusions under the control of the same promoter, to allow direct selection of plants expressing the insecticidal protein at a high level, were also examined. The levels of the insecticidal protein detected by ELISA were also very low in the plants, expressing the intact gene. The transgenic plants, containing the truncated or fusion constructs, expressed higher levels of the insecticidal protein, although the *cry* gene RNA was not reliably detected by Northern blot. Ribonuclease protection assays using the first 186 bases of insecticidal protein coding sequence as a probe indicated that RNA levels were 10-fold lower than the protein levels in these plants. Regardless of the low detected insecticidal protein levels the transgenic plants showed good protection towards the first instar larvae of *Manduca sexta* in laboratory and greenhouse bioassays.

Fischhoff et al. [21] generated transgenic tomato plants containing truncated *cry 1Ab* gene under the control of CaMV 35S promoter. The polyadenylated transcripts of the predicted size were detected in the callus tissues from transgenic plants, although the insecticidal protein levels were too low to be detected by immunoassay.

These tomato plants, and the others containing *cry 1Ab* gene under the control of the mannopine synthase promoter [22] in different tomato cultivars were reported to be tolerant to tobacco hornworm, tomato fruitworm (*Heliothis zea*), and tomato pinworm (*Keiferia lycopersicella*), though the Cry 1Ab protein was immunochemically undetectable.

Inducible expression of the insecticidal protein gene from *B. thuringiensis kurstaki* HD 1 have been reported in transgenic tobacco plants containing a truncated insecticidal protein gene from *B. thuringiensis* under the control of the *PR 1a* promoter [23]. The homozygous transgenic lines were selected and tested for their resistance towards *M. sexta* larvae. The transgenic lines were found to be tolerant towards tobacco hornworm larvae after induction with salicylic acid, and the *cry 1Ab* gene specific RNA was detected 14 and 21 days after induction, although the insecticidal protein level was too low to be detected by ELISA.

Carozzi et al. [24] examined the developmental and tissue specific expression during the course of field tests in tobacco plants containing a truncated *cry 1Ab* gene with the optimized start codon under the control of *CaMV 35S* promoter and *CaMV 35S* terminator. They showed that the levels of insecticidal protein and mRNA were increased throughout the plant development with a pronounced rise in the expression at the time of flowering. The insecticidal protein gene expression was detected by ELISA in most of the tissues with the predominance in old tissues.

The truncated genes from the *cry 1A* family have been also expressed in transgenic potato [25], *Populus nigra* [26], and chrysanthemum [27]. Although the transgenic plants showed resistance towards *Manduca sexta* and other susceptible insect species, the common features in all the cases of expression of intact and truncated natural *cry* genes in transgenic plants were very low level of degraded polyadenylated RNA transcripts, which was 100 times lower than the reporter genes, and correlated with the low levels of insecticidal protein.

Murray et al. [28] investigated the possible causes for the poor expression of the insecticidal protein genes in transgenic plants. They found, by experiments with transgenic tobacco plants and electroporated carrot protoplasts, that the main cause of the poor expression was due to instability of *cry* transcripts in plant cells, and supposed that it was determined by instability sequences contained in the gene coding region. They also proposed several possible mechanisms which may contribute to this instability, including premature polyadenylation, splicing using cryptic splice sites within the gene and improper codon usage for expression in plants which may cause ribosome pausing leading to rapid 3'-5' or 5'-3' or both directions of RNA metabolism.

Recently it was reported that low cytoplasmic steady-state *cry 1Ab* mRNA levels in transgenic tobacco plants were a result of inefficient transcript formation in the nucleus and marginal import flow to the cytoplasm rather than the rapid turnover of the message in the cytoplasm [29]. The *in vitro* synthesized *cry 1Ab* mRNA introduced into tobacco protoplasts was surprisingly stable, but the splicing of three cryptic introns was found to occur by RT-PCR of nuclear RNA.

Perlak et al. [30] demonstrated that modifications of the truncated *cry 1Ab* and *cry 1Ac* coding sequences by introducing translationally neutral mutations enhanced the expression of these genes in tobacco and tomato plants up to 100-fold with totally synthetic gene compared to the wild type. The basic principles for these modifications included replacement of the regions resembling plant introns and polyadenylation sites, removal of ATTTA sequences which were shown to destabilize mRNA in mammalian and plant systems [31] and finally replacement of the rarely used codons in plant genes to more plant-like ones. Following these basic principles, a number of synthetic insecticidal protein genes were constructed and expressed in transgenic cotton [22], potato [32, 33], maize [34], and rice [35]. Typically the transgenic plants produced the insecticidal protein to levels of 0.01 to

0.5% of the total leaf protein which was at least 100 fold higher than the wild type gene. The transgenic plants expressing the synthetic *cry* genes were reported to effectively control economically important pests such as Colorado potato beetles [32, 33], cotton bollworm (*Heliothis zea*) [22] and European corn borer [34], which are rather resistant to the Bt toxin. However, similar insecticidal protein expression levels were not sufficient to completely protect the transgenic rice plants from the leaf folder (*Cnaphalocrocus medinalis*) [35]. The characteristics of the synthetic Bt toxin genes expressed in plant cells are summarized in Table 1-2.

Additional enhancement of the expression level of the insecticidal protein genes was reported by Wong et al. [36]. They showed the transgenic tobacco plants expressing synthetic *cry 1Ac* gene under the control of CaMV 35S promoter with duplicated enhancer region combined with RuBisCo small subunit untranslated leader and transit peptide accumulated the insecticidal protein at level up to 1% of the total leaf protein.

Recently McBride et al. [37] proposed a new approach to achieve a high level of insecticidal gene expression in transgenic tobacco. They demonstrated that the introduction of the intact unmodified *cry 1Ac* gene under the control of chloroplast specific promoter into chloroplast genome of tobacco plants resulted in accumulation of 3-5% of the total soluble protein as a protoxin. The transgenic plants expressing such a high level of the insecticidal protein were extremely toxic to *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua*. However, the applicability of such method at present is limited only to tobacco plants.

Table 1-2 Characteristics of synthetic Bt toxin genes expressed in plant cells\*

| Gene           | Lenght <sup>1</sup> | Change in GC (%) | Codons Changed | Plant Species | Expression                  |                              |                                 | Active Against          | Reference |
|----------------|---------------------|------------------|----------------|---------------|-----------------------------|------------------------------|---------------------------------|-------------------------|-----------|
|                |                     |                  |                |               | Increased mRNA <sup>2</sup> | Maximum Protein <sup>3</sup> | Increased Activity <sup>4</sup> |                         |           |
| <i>cry 1Aa</i> | 1-645               | Nr <sup>5</sup>  | 141            | poplar        | nr                          | nr                           | nr                              | forest tent caterpillar | [38]      |
| <i>cry 1Ab</i> | 1-612               | nr               | nr             | cotton        | nr                          | 0.1 %                        | +                               | gypsy moth              | [22]      |
|                |                     |                  |                |               |                             |                              | +                               | cabbage looper          |           |
|                |                     |                  |                |               |                             |                              | +                               | beet armyworm           |           |
| "              | 1-615               | 37 to 49         | 356            | tomato        | +                           | 0.2-0.3 %                    | +                               | cotton bollworm         | [30]      |
|                |                     |                  |                | tobacco       | nr                          | 0.2-0.3 %                    | +                               | tobacco hornworm        |           |
| "              | 29-607              | 37 to 41         | 55             | tomato        | +                           | 0.02 %                       | +                               | tobacco hornworm        |           |
|                |                     |                  |                | tobacco       | nr                          | 0.02 %                       | +                               | tobacco hornworm        | [30]      |
| "              | 1-648               | 38 to 65         | 549            | maize         | nr                          | 0.4 %                        | +                               | European corn borer     | [34]      |
| "              | 1-724               | 38 to 59         | 482            | rice          | nr                          | 0.05                         | nr                              | striped stem borer      | [35]      |
|                |                     |                  |                |               |                             |                              | nr                              | leaffolder              |           |
| "              | 1-646               | nr               | 21             | tomato        | +                           | <<6                          | +                               | tobacco hornworm        | [39]      |
|                |                     |                  |                | tobacco       | +                           | <<                           | +                               | tobacco hornworm        |           |
| "              | 1-648               | 38 to 65         | 549            | rice          | +                           | 0.009 %                      | +                               | yellow stem borer       | [40]      |
|                |                     |                  |                | rice          | +                           | 0.009 %                      | +                               | striped stem borer      |           |
| "              | nr                  | nr               | nr             | rice          | +                           | 3 % ?                        | +                               | yellow stem borer       | [42]      |
|                |                     |                  |                | rice          | +                           | 3 % ?                        | +                               | striped stem borer      |           |
| <i>cry 1Ac</i> | 1-640               | nr               | nr             | cotton        | nr                          | 0.1 %                        | +                               | cotton bollworm         | [22]      |
|                | 1-615               | nr               | 306            | cotton        | nr                          | 0.1 %                        | +                               | cotton bollworm         | [22]      |
|                |                     |                  |                | tomato        | nr                          | 0.2-0.3 %                    | nr                              | tobacco hornworm        | [30]      |
| "              | 29-615              | nr               | nr             | tobacco       | nr                          | 0.2-0.3 %                    | nr                              | tobacco hornworm        | [30]      |
| "              | 10-613              | 34 to 64         | 496            | maize         | +                           | nr                           | +                               | nr                      | [43]      |
|                |                     |                  |                | tobacco       | +                           | nr                           | +                               | tobacco hornworm        |           |
|                |                     |                  |                | Arabidopsis   | +                           | nr                           | +                               | tobacco hornworm        |           |
| "              | 1-618               | 34 to 45         | 364            | rice          | +                           | 0.025 %                      | +                               | yellow stem borer       | [41]      |
|                |                     |                  |                | rice          | +                           | 0.025 %                      | +                               | striped stem borer      |           |
| "              | nr                  | nr               | nr             | rice          | +                           | 3 % ?                        | +                               | yellow stem borer       | [42]      |
|                |                     |                  |                | rice          | +                           | 3 % ?                        | +                               | striped stem borer      |           |

Table 1-2 (continued) Characteristics of synthetic Bt toxin genes expressed in plant cells\*

| Gene          | Lenght <sup>1</sup> | Change in GC (%) | Codons Changed | Plant Species | Expression                  |                              |                                 | Active Against                            | Reference |
|---------------|---------------------|------------------|----------------|---------------|-----------------------------|------------------------------|---------------------------------|-------------------------------------------|-----------|
|               |                     |                  |                |               | Increased mRNA <sup>2</sup> | Maximum Protein <sup>3</sup> | Increased Activity <sup>4</sup> |                                           |           |
| <i>cry 1C</i> | 1-654               | nr               | 42             | tobacco       | +                           | <<                           | +                               | beet armyworm<br>tobacco hornworm         | [39]      |
| “             | 1-630               |                  | 249            | tobacco       | +                           | 0.2 %                        | +                               | Egyptian cotton leafworm                  | [44]      |
|               |                     |                  |                | alfalfa       | +                           | 0.2 %                        |                                 | beet armyworm                             |           |
|               |                     |                  |                | Arabidopsis   | +                           | nr                           | nr                              | Egyptian cotton leafworm<br>beet armyworm |           |
| <i>cry 3A</i> | 48-644              | 36 to 49         | nr             | tobacco       | nr                          | 0.6 %                        | nr                              | nr                                        |           |
| “             | 48-645              | 37 to 49         | 347            | potato        | nr                          | 0.3%                         | +                               | colorado potato beetle                    | [45]      |
| “             | 1-610               | 37 to 45         | 190            | potato        | nr                          | 0.025                        | nr                              | colorado potato beetle                    | [32]      |
|               |                     |                  |                | carrot        | +                           | 0.005                        | nr                              | nr                                        |           |
|               |                     |                  |                | maize         | +                           | 0.005                        | nr                              | nr                                        |           |

\* After [46], modified and updated

<sup>1</sup> Amino acid residues from protoxin genes included in modified Bt toxin genes

<sup>2</sup> + indicates increase in mRNA levels of modified gene relative to the wild type gene

<sup>3</sup> Highest observed level of Bt toxin accumulation as % of total protein

<sup>4</sup> + indicates increase in insecticidal activity resulting from expression of modified gene relative to the wild type gene

<sup>5</sup> nr not reported

<sup>6</sup> << Below limit detection

? not reliably quantified

### **1.3 PURPOSE OF THE STUDY**

Members of *Spodoptera* genus (*Lepidoptera*, *Noctuidae*) are generalist caterpillars called leafworms and cutworms, widely spread in tropical and subtropical areas which feed on over 40 different plant families and may cause over 80 percent defoliation in heavily infested crops [47]. These are particularly difficult to control with chemical insecticides due to developed resistance to organophosphorus and pyrethroid insecticides. A large number of transgenic crops expressing mainly Cry 1A type Bt toxins have been generated (Table 1-2) and some of them are readily commercially available for insect control. Although very efficient for the control of some *Lepidoptera* species of economic importance, the Bt crops currently available on the market are still inefficient for control of the more resistant *Lepidoptera* species such as *Spodoptera litura* [22]. Based on the published data, it appears that there are two main reasons for the low efficiency of the commercial crops for control of the *S. litura*, relatively high activity of the gut proteases of the larvae of this species [48], and lower specificity of the Cry 1A toxins to the receptors on the midgut epithelial cells of *S. litura* [49, 50].

Two approaches to generate transgenic tobacco plants expressing genetically engineered Bt toxin genes with improved efficiency for control of *S. litura* are proposed in the present study. The first approach addresses the modification of protease sensitivity of the Cry 1Ab proteins. It is based on expression of truncated *cry 1Ab* gene with a mutated putative protease cleavage site. The second approach is based on the expression of the active fragment of a *cry 1C* gene which is the most active towards *Spodoptera* species among Bt toxins known to date. Although expression of different classes of Bt toxins was attempted under the control of various promoters (see the literature review), there is no information on the green tissue specific and light inducible expression of *cry 1C* genes in transgenic plants. Therefore, I attempted expression of the *cry 1C* gene from *B.*

*thuringiensis* under the control of *rbcS* promoter from *N. plumbaginifolia* in tobacco plants.

In Chapter II it was attempted to engineer transgenic tobacco plants resistant to *S. litura* by the expression of a truncated mutant *cry 1Ab* gene. The mutant protoxin in which three amino acids, Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>637</sub>, were replaced with Gln by site directed mutagenesis was 2.5 times more active towards *S. litura* than the wild type protein when expressed in *E. coli* [51].

The truncated mutant and wild type *cry 1Ab* genes coding for the first 647 amino acids were each placed under the control of *CaMV* 35S promoter and *nos* terminator in a binary vector, and were transformed into a haploid clone of *N. tabacum* cv. Samson nn via *Agrobacterium*. The expression of the mutant *cry 1Ab* gene will clarify the question whether modifications of the potential protease attack sites in the protein sequence of the Cry 1A type Bt toxins is useful for improvement of the expression level and resistance of transgenic tobacco plants towards rather resistant *Spodoptera* species.

In Chapter III engineering of transgenic tobacco plants resistant to *S. litura* by tissue specific expression of the *cry 1C* gene from *B. thuringiensis* was attempted. A truncated *cry 1Ca5* gene coding for the active fragment (N-terminal 655 amino acids) was placed under the control of a tobacco *rbcS* promoter, which is one of the strongest green tissue specific promoters, and was transformed into tobacco plants. The transgenic tobacco plants transformed with the wild type gene were characterized by PCR, RT-PCR and insect bioassays with the first instar of *S. litura* larvae. The green tissue specific expression of the *cry 1C* gene will bring new insights on the tissue specific expression of *cry 1C* genes in tobacco plants. It will also provide additional information on the usefulness of the green tissue specific expression as a delivery system for the Cry 1C protein to the major feeding site of *S. litura* larvae, to control this insect species by transgenic crops approach.

The fusion gene between *rat CYP1A1 cytochrome P450* and *yeast P450 oxidoreductase (CYP1A1/YR)* which was shown to confer resistance to the herbicide chlorotoluron in transgenic tobacco plants [52]. The *CYP1A1/YR* gene, placed under the control of CaMV 35S promoter, was also cotransformed with the *cry 1C* gene directed by the *rbcS* promoter from *N. plumbaginifolia*. The kanamycin resistant transgenic tobacco plants were subsequently selected for *in vitro* resistance to the herbicide chlorotoluron. The coexpression of *CYP1A1/YR* and the *cry 1C* genes will combine two very useful agronomic traits such as insect and herbicide tolerance in transgenic tobacco plants. On the other hand, it will provide an additional selectable marker for tobacco plants cotransformed with both genes. If, the plants selected *in vitro* for higher resistance to the herbicide chlorotoluron, show higher insecticidal effects, this will be the first direct evidence that the *CYP1A1/YR* gene is a useful *in vitro* distal selection marker in tobacco transformation.

From the literature review it is clear that the expression levels of the Bt proteins, high enough to effectively control the economically important insect species could not be achieved with the native genes despite various promoter and terminator sequences used in each study, suggesting that the problems with the low mRNA and protein accumulation are located onto the coding sequences of the Bt genes. Two recent studies [29, 53] clearly demonstrated that the problem was posttranscriptional. Splicing at cryptic sites within the *cry 1Ab* coding region was found to limit the *cry 1Ab* expression in tobacco plants [29] and in the case of the *cry 1Ac* gene cryptic polyadenylation appeared to be more limiting to the gene expression in tobacco and *Arabidopsis thaliana* plants [53]. However, no information is available in the literature on what are the factors, limiting the expression of *cry 1C* genes in transgenic plants. Previous attempts to improve the expression levels of a truncated *cry 1C* gene by targeted modification of the motifs, suspected to cause mRNA instability [39] showed

marginal improvement in the expression levels suggesting that additional destabilizing factors which were difficult to predict were present in the gene coding region. Such motifs were apparently removed in a fully reconstructed *cry 1C* gene [44] which expressed under the control of CaMV 35S promoter with four enhancer regions showed significant protein accumulation in tobacco and alfalfa plants, sufficient to reliably control *S. exigua* species. Another recent study [43] clearly demonstrated that the sequences interfering with mRNA stability in transgenic *Arabidopsis* plants were dispersed throughout the coding sequence of a truncated *cry 1Ac* gene. Therefore, I attempted to optimize the coding sequence of the truncated *cry 1C* gene for high level green tissue specific expression in transgenic tobacco plants by a total synthesis of the gene. The reconstruction strategy involved a switch of the codon usage preference in the reconstructed gene to resemble that of the dicotyledonous *rbcS* genes which are highly expressed in green tissues and have pronounced codon usage bias compared to the bulk of dicotyledonous genes [54]. Transgenic tobacco plants expressing high levels of Cry 1C protein in the leaf tissues, which are the primary target for the *Spodoptera* larvae will be beneficial in agriculture for sustainable control of this traditionally difficult to control insect species by transgenic crops approach. Moreover, as long as, the Cry 1C toxins bind to receptors on the insect midgut, different from the receptors for cry 1A type toxins, transgenic crops expressing high levels of Cry 1C protein in a tissue specific manner may become an important part of the insect resistance management strategies of the near future

## **Chapter II**

**Expression of genetically engineered cry  
1Ab genes from Bacillus thuringiensis ssp.  
aizawai in tobacco plants**



## **2.1 INTRODUCTION**

Most of the current commercially available transgenic Bt crops express a single Cry 1Aa, Cry 1Ab or Cry 1Ac protein from *B. thuringiensis*. Although very effective towards other *Lepidoptera* species the transgenic plants expressing Cry 1A toxins even at high levels are not sufficient to control the more resistant *Spodoptera* species. Low sensitivity of the *Spodoptera* larvae to the Bt toxins is at least in part determined by the high activity of the midgut proteases of these insect larvae [48].

A mutant version of Cry 1Ab protoxin in which Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>629</sub> were replaced by Gln was produced by site directed mutagenesis [51]. The mutant protein was 2.5 fold more toxic to *S. litura* larvae than the wild type and showed different processing pattern by *S. litura* midgut proteases [51].

The present study attempted engineering of resistance to *Spodoptera litura* larvae in transgenic tobacco plants by expressing each of the truncated mutated and the wild type *cry 1Ab* gene versions and to compare insecticidal effects of these transgenic plants towards the first instar of *Spodoptera litura* larvae.

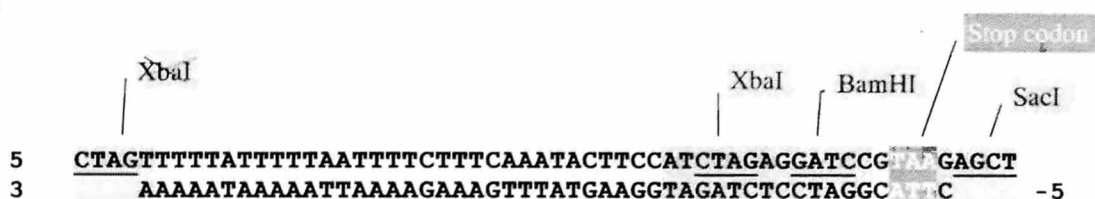
## **2.2 MATERIALS AND METHODS**

### **2.2.1 Construction of recombinant plasmids**

All recombinant DNA procedures were done according to Sambrook et al. [55] unless otherwise mentioned. The vector pBURX1 was constructed on the base of the binary plasmid pBI121 by replacement of the coding sequence of GUS reporter gene between CaMV 35S promoter and nopaline synthase (Nos) terminator with a synthetic linker as shown on Fig. 2-1A. The linker was prepared by annealing of two complementary oligonucleotides to form compatible XbaI and SacI ends. The internal sequence of this linker contained alfalfa mosaic virus (AMV) RNA4 untranslated region, XbaI and BamHI cloning sites, and a termination codon adjusted downstream to the BamHI site (Fig. 2-1A). The linker was linked to the unique XbaI and SacI sites in pBI121 to form pBURX1. The XbaI site was disrupted upon ligation (Fig. 2-1A), so that the XbaI site, contained in the synthetic linker remained unique in the constructed vector.

The truncated versions of the native wild type and triple mutant *cry 1Ab* genes of *B. thuringiensis* strain *aizawai* IPL7 were excised as 2.0 kb BamHI/BclI fragments from the plasmids pTB1 (wild type) and pADC3 (triple mutant) [51] which contained the coding region for the amino acids 1 to 645 of Cry 1Ab protein. These fragments also included a 72 bp. 5' flanking region containing a putative RBS site from *B. thuringiensis* [56]. Furthermore each of these BamHI/BclI fragments was inserted into the BamHI site of pBURXI to yield the plasmids pBTTW and pBTM (Fig 2-1 B).

(A)



(B)

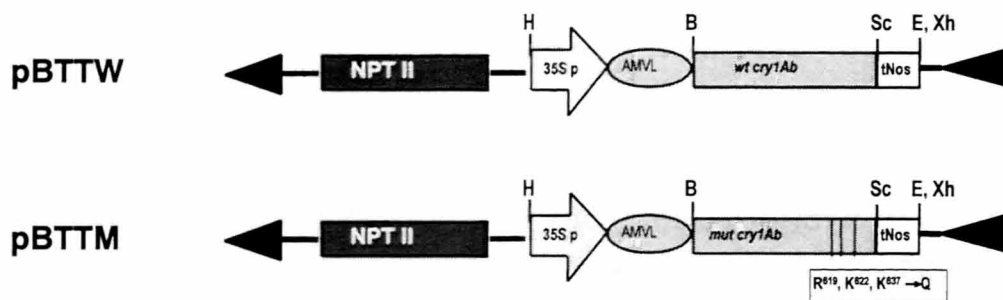


Fig. 2-1. Schematic representation of the recombinant plasmids for the expression of the truncated wild type and mutant *cry 1Ab* genes in tobacco plant.

(A): Sequence of the Alfalfa Mosaic Virus RNA4 untranslated leader (AMVL);

(B): Structure of T-DNA regions of the recombinant plasmids (not in scale). AMVL, AMV RNA4 UTR; wt, wild type; mut., mutant; tNos, NOS 3' UTR; LB, left T-DNA border; RB, right T-DNA border; 35Sp, CaMV 35S promoter; ; B, Bam HI; E, Eco RI; H, Hind III; Sc, Sac I; X, Xho I

### **2.2.2 Tobacco transformation**

Each of the plasmids pBTTW and pBTM was introduced into *Agrobacterium tumefaciens* strain LBA4404 by Ca Cl<sub>2</sub> method [57]. Haploid tobacco *Nicotiana tabacum*, cv. Samson nn. originated from microspore culture and maintained *in vitro* was used for transformation. *Agrobacterium* leaf disc transformation was done essentially as described by Horsh, et al [58]. The regenerated shoots were selected on a medium, containing 100 mg/l kanamycin and maintained *in vitro* on a kanamycin containing medium.

### **2.2.3 Southern blot**

Total DNA was extracted from a young leaf tissues of *in vitro* grown plants as described by Delaporta et al [59]. Ten micrograms Bam HI digested DNA samples were loaded for electrophoresis. DNA was transferred to a Hybound N<sup>®</sup> membrane (Amersham, London, England) and hybridized to a DIG PCR labeled 1.5 kb fragment of the *cry 1Ab* gene according to the vendor's instructions. Detection was done with DIG labeling and detection kit (Boehringer Mannheim, Heidelberg, Germany) according to the manufacturers instructions.

### **2.2.4 Genomic PCR**

Genomic DNA was extracted from 0.1 g leaf tissue by the method of Delaporta [59].

PCR was done with 0.5 µg genomic DNA with rTaq (Takara, Tokyo, Japan) in 50 µl reactions 1X buffer 2 mM dNTPs, rTaq enzyme, 50 pM of each sense primer:

5'-CCCTTACAACCGCTATTCCT-3' and the antisense primers:

5'-TTCACCGCCTTTTGTGCTCT-3' (pBTTW) and:

5'-TTCACCGCTTGTGCTTG-3' (pBTM)

which amplify a 1.5 kb. fragment of the wild type and the mutant *cry 1Ab* gene respectively. Amplification was done for 30 cycles at 95 °C 1

min, 55 °C 2 min. and 72 °C 3 min and 10 µl of the PCR reaction were analyzed by electrophoresis in 1% TAE agarose gel.

#### **2.2.5 NPT II activity gel assay**

The NPT II assay was essentially done according to Angenon et al. [60]. Thirty micrograms of total leaf protein were resolved in native 10% PAGE. The reaction mixture containing 1.5 mg kanamycin sulfate and 70 µl [ $\alpha$ -<sup>32</sup>P] ATP was mixed with 35 ml melted 1% agarose and poured over the PAGE gel surface. After 30 min. incubation at 37 °C the reaction products were blotted onto Whatman® P-81 paper followed by extensive washing in phosphate buffer. The radiation emission was quantified on a BAS 2000 Image analyzer (Fuji Film, Tokyo, Japan)

#### **2.2.6 RT-PCR experiments**

polyadenylated RNA was isolated from 0.1 g of leaf tissue by using micro mRNA purification kit (Pharmacia, Upsala, Sweden). The first strand cDNA was synthesized from 200 ng polyadenylated RNA with the first strand cDNA synthesis kit (Pharmacia) and the provided oligo dT 18 bifunctional primer following the manufacturers instructions. Five microliters of the first strand reactions were amplified with the primers forward:

5'-GACCAGGATTTACAGGAGATATTCT-3' (N1), and reverse:

5'-TGGAAGAAGTAAACAGCTCATTCA-3' (N1) amplifying 422 bp. at the 3' end of the truncated *cry 1Ab* gene. The PCR was carried on with rTaq (Takara) in the provided buffer for 30 cycles at 95°C for 30 sec., 55°C for 30 sec and 72°C for 1 min. Ten µl of the reactions were analyzed in 5% TBE PAGE. The amplified fragments were extracted from the PAGE gel in TE buffer and blunt cloned into Sma I site of pUC 19 plasmid. The cloned RT-PCR fragments were sequenced by fluorescent dye terminator method on Shimazu DSQ-1 Sequencer.

### **2.2.7 Bioassay with *Spodoptera litura* larvae**

Five freshly hatched larvae of *S. litura* per detached leaf from control and transgenic plants were infested in three replications on moistened filter paper in petri dishes and incubated at 16/8h light/dark cycle 15, 000 lux , 25 °C 65% RH.

At the end of the experiment (after nine days) the mortality was recorded and the weights of individual survived insect larvae were measured on a electronic microbalance .

## **2.3 RESULTS AND DISCUSSION**

### **2.3.1 Recombinant plasmid constructs**

Site directed mutagenesis experiments with the native 135 kD Cry 1Ab protoxin from *B. thuringiensis* ssp. *aizawai* IPL7 and its expression in *E. coli* revealed that a mutant with triple amino acid replacements Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>637</sub> to Gln which are located close to the putative activation site was 2.5-fold more active towards *S. litura* larvae than the wild type protein [51]. Truncated versions coding for the first 645 amino acids of this mutant gene and the original wild type protein gene were used for the construction of the recombinant plasmids pBTTW (wild type) and pBTTM (mutant) (Fig 2-1B).

The 5' untranslated regions for the insecticidal protein genes were reconstructed by addition of a synthetic linker containing AMV RNA4 untranslated leader sequence (Fig. 2-1A), 5' of the 72 bp bacterial 5' untranslated region which was present in the 2 kb BamHI/BclI fragments, and a termination codon adjusted to the reading frame of the truncated *cry 1Ab* coding region at the 3' end of the gene. Placement of viral untranslated leader sequences 5' of the coding sequence of reporter genes have been shown to improve

transgene steady state mRNA and protein levels in transgenic plants [61].

### **2.3.2 Southern blot analyses**

Both recombinant plasmids were transformed in a haploid tobacco Samson nn. The haploid line was chosen because of its high regeneration ability and because the transgenes can be easily made homozygous by dihaploidization. Forty kanamycin resistant pBTTW (BTW) and 32 pBTTM (BTM) plants were regenerated and 85 % of them were positive for the presence of the *cry 1Ab* gene by PCR. The presence and integrity of the transgene was confirmed by Southern blot (Fig. 2-2). Digestion with Bam HI and hybridizing with a 1.5 kb. probe covering 2/3 of the *cry 1Ab* gene coding sequence allowed estimation of the copy number because this enzyme recognizes a single site in the T-DNA region (Fig. 2-1). Most of the transgenic plants tested contained single transgene copy lanes 1, 4, 6, 7, 8, 9 (Fig. 2-2). Although transgenic plants with two to five copies were found c. a. lanes 2, 3, 6 (Fig. 2-2), the higher copy number in these plants did not correlate with improved insecticidal activity.

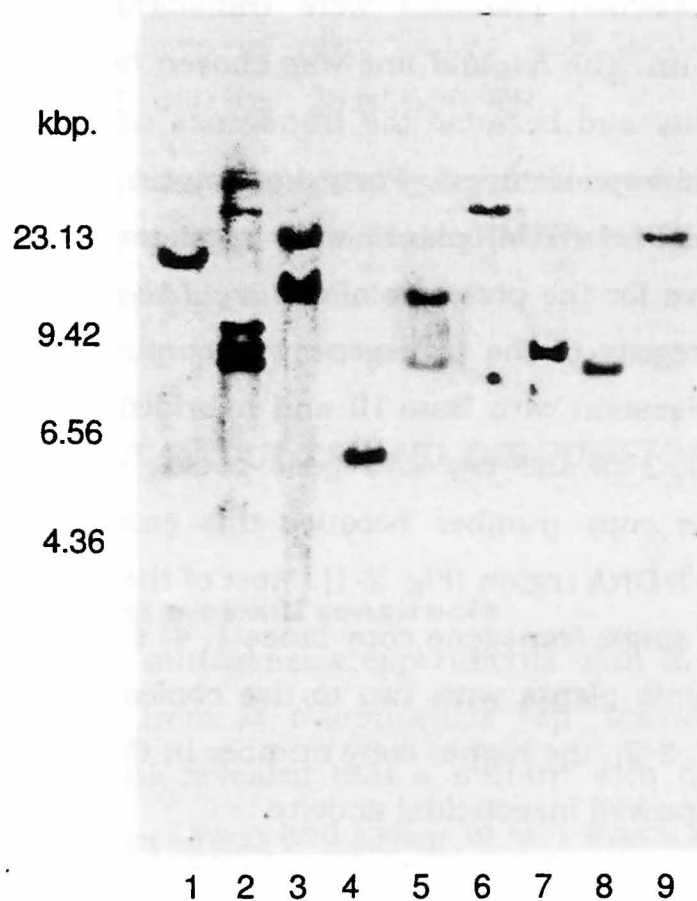
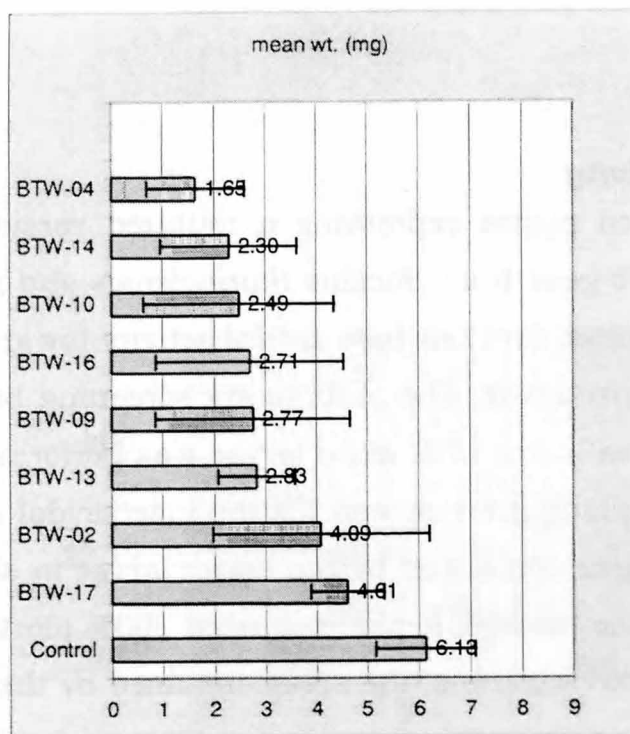


Fig. 2-2. Southern blot of BamHI digested total DNA from transgenic tobacco plants expressing the mutant and the wild type truncated *cry IAb* genes.

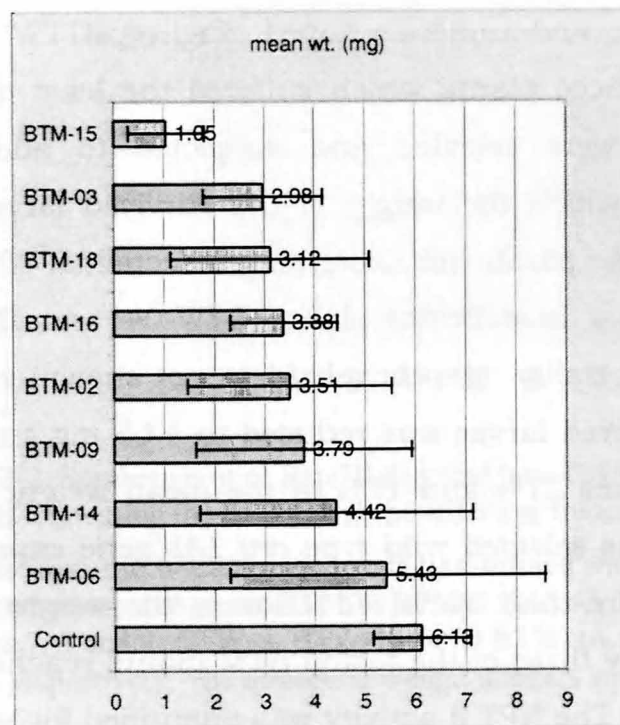
Lanes from 1 to 6- transgenic plants transformed with plasmid pBTTM containing the mutant *cry IAb* gene: BTM-1, BTM-2, BTM-3, BTM-5, BTM-7, and BTM-9. Lanes 7, 8 and 9- BTW-8, BTW-13 AND BTW-16, expressing wild type *cry IAb* gene respectively. The molecular weight markers are shown on the left.

### **2.3.3 Insecticidal activity**

Transgenic tobacco plants expressing a mutated version of a truncated native *cry 1Ab* gene from *Bacillus thuringiensis* and its wild type counterpart were tested for their insecticidal activity towards first instar of *Spodoptera litura* larvae. The preliminary screening bioassay experiments with the first instar of *S. litura* larvae was performed and the transgenic tobacco plants were scored for the insecticidal activity on the base of the leaf area consumed by the insect larvae in six days trial. Although none of the transgenic plants caused 100% mortality of the larvae in nine days feeding trials, the area consumed by the larvae was significantly reduced in transgenic leaves compared with the control and survived larvae fed on the leaf tissue from some of the plants appeared smaller and underdeveloped. Eight pBTTW and 8 pBTM transformed tobacco plants which suffered the least damage by the insect feeding were selected and subjected to additional bioassay experiment in which the weight of the survived larvae was measured as more precise parameter describing insecticidal effect. In this experiment the most insecticidal plants BTW-04 and BTM-15 caused 87% and 73% mortality respectively (data not shown) and the mean weight of the survived larvae was reduced to 1.65 mg and 1.05 mg (Fig. 2-3) which makes 27% and 17% of the mean weight of the control larvae. Five of the selected wild type *cry 1Ab* gene expressing plants (BTW) caused more than 50 % reduction in the weight of the survived larvae while only three of the tested BTM plants reached this level of toxicity (Fig. 2-3). The NPT II activity was quantified for some of the selected insecticidal transgenic tobacco plants (Fig. 2-4).



**Tobacco plants transformed with pBTW**



**Tobacco plants transformed with pBTM**

**Fig. 2-3. Insecticidal activity of the wild type (BTW) and a mutant (BTM) *cry 1Ab* expressing tobacco plants towards neonate *Spodoptera litura* larvae.**

The detached leaf bioassay was performed with five freshly hatched larvae and leaf tissue from hidroponically grown plants in three replications. The results were scored after nine days incubation at 25°C 16/8 h light/dark cycle without replacement of the leaf tissue. "mean wt.", mean weight of the survived larvae in miligram. Error bars represent the standard deviation between replications of the experiment.

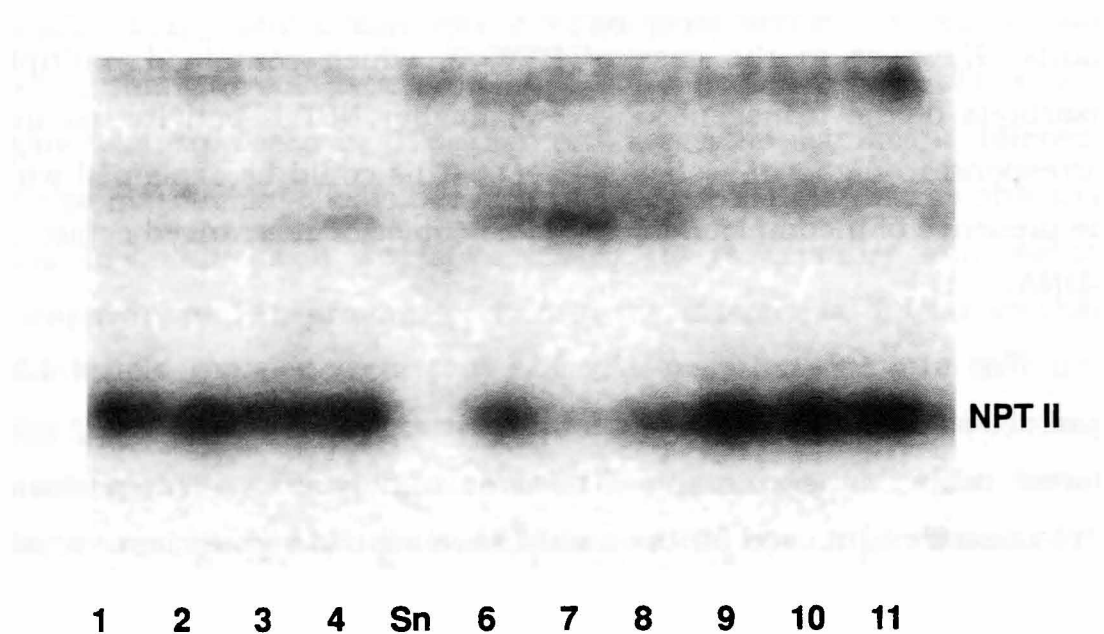


Fig. 2-4. *In situ* NPT II activity of total leaf protein extracts from the transgenic tobacco plants.

Lanes 1-4 , tobacco plants transformed with the plasmid pBTTW: BTW 2, BTW 14, BTW 16 and BTW18; lane 5, untransformed control; lanes 6-12, plants transformed with the plasmid pBTTM: BTM 2, BTM 4, BTM 5, BTM 10, BTM 15, BTM 18 and BTM 19 respectively

The activity stained band corresponding to the size of NPT II protein was detected in all tested transgenic plants and was not present in the untransformed control plants (Fig. 2-4). The additional bands with the higher molecular weight showing NPT II activity resulted probably from NPT II protein aggregates. In most of the cases The higher NPT II activity was in agreement with the insecticidal effects of the transgenic plants. However in the case of BTW 2, which contained multiple insertions of the transgene (Fig. 2-2), higher NPT II activity did not correspond to higher insecticidal effects. This could be explained with the presence of incomplete *cry 1Ab* gene copies or rearranged copies of T-DNA.

The attempts to detect Cry 1Ab protein by western blot of 100 µg of crude leaf protein extracts from the transgenic plants and 1: 500 diluted rabbit antiserum raised towards wild type Cry 1Ab protoxin were unsuccessful even for the plants showing the highest insecticidal activity. Although the Cry 1Ab protein level was below the detection limit of Western blot, which for the antibody used was 10 ng/100µg protein, the plants expressing each of the *cry 1Ab* genes showed significant inhibition of the insect growth reaching up to 73 % and 83 % for the best plants BTW4 and BTM15 respectively. Most of the plants which caused more than 50 % reduction of the larval weight killed more than 50% of the larvae. Based of the LC<sub>50</sub> value of Cry 1Ab protoxin for neonate larvae of *S. exigua* which was estimated between 100 and 200 µg per gram diet [50], similar concentrations of the protein per gram tissue could be expected in these plants. Several research groups had produced transgenic plants expressing natural *cry 1Ab* genes [19, 21, 25, 62]. However in only one of them [62] testing of the transgenic plants expressing an unmodified *cry 1Ab* gene towards *Spodoptera* species was attempted. Transgenic cotton was tested towards *Spodoptera exigua* (beet armyworm) and the transgenic plants showed only marginal protection towards the insect damage but no mention was made about the actual levels of

insecticidal activity. The same plants were well protected towards tobacco budworm (*Manduca sexta*), which was 800 fold more sensitive than *Spodoptera exigua* to Cry 1Ab, though the insecticidal protein levels were below the detection limit of the Western blot [62].

The Cry 1Ab protoxin with triple amino acid replacements Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>637</sub> was 2.5-fold more active towards *S. litura* larvae than the wild type protoxin [51]. Truncated version of the same gene was expressed in BTM plants, however no significant difference in the insecticidal effects between BTW and BTM plants was observed. The low expression levels prevented us to quantify and directly compare Cry 1Ab protein levels and the insecticidal effects in these plants. The only indirect evidence that was observed was that BTW plants with the same levels of insecticidal activities showed lower levels of NPT II activity than the BTM counterpart (data not shown). So, we do not exclude the possibility that the truncated version of the mutant protein is less stable than the wild type when expressed in transgenic plants.

#### **2.3.4 RT-PCR detection of spliced *cry 1Ab* transcripts.**

The *cry 1Ab* specific transcripts with the expected size were detected in Northern blot of 5 µg mRNA from the transgenic plants (data not shown). However additional smear of shorter or degraded transcripts appeared on the blot. Therefore the presence of the *cry 1Ab* transcripts was confirmed by the more sensitive RT-PCR technique. The first strand was synthesized using a oligo dT18 primer followed by a PCR with primers N1 and N2 which amplify a region of 422 bp. (1472-1893 relative to translation start position) at the 3' end of the truncated *cry 1Ab* gene. In addition to the expected band of 422 bp. two shorter PCR products of 349 and 214 bp. appeared on the electrophoresis (Fig. 2-5A). Subsequent cloning and sequencing of the above mentioned shorter products revealed that those originated from *cry 1Ab* transcripts in which two small introns of 73 bp. and 208 bp

were spliced from cryptic splicing sites in the *cry 1Ab* gene coding sequence (Fig. 2-5B).

Low transgene mRNA levels and low or undetectable insecticidal protein levels were also reported by the other groups who generated transgenic plants expressing native bacterial *cry* genes [18, 21, 28, 63] and poor accumulation was attributed to mRNA instability. This view was supported by the reconstruction of codon optimized Bt genes in which codon bias was altered to more plant-like and potential RNA destabilizing factors were removed. The use of such reconstructed genes in transformation experiments directed 100 fold higher Bt protein expression compared to wild type genes in transgenic plants and complete control of the target insect species [30, 32, 33, 35, 44]. Van Aarsen, R et al. [29] clearly demonstrated that the problem was posttranscriptional by comparison of transcriptional activities of *cry 1Ab* gene and the cotransformed *npt ii*. Nuclear *run-on* transcription experiments showed that both transgenes were transcribed with the same efficiency.

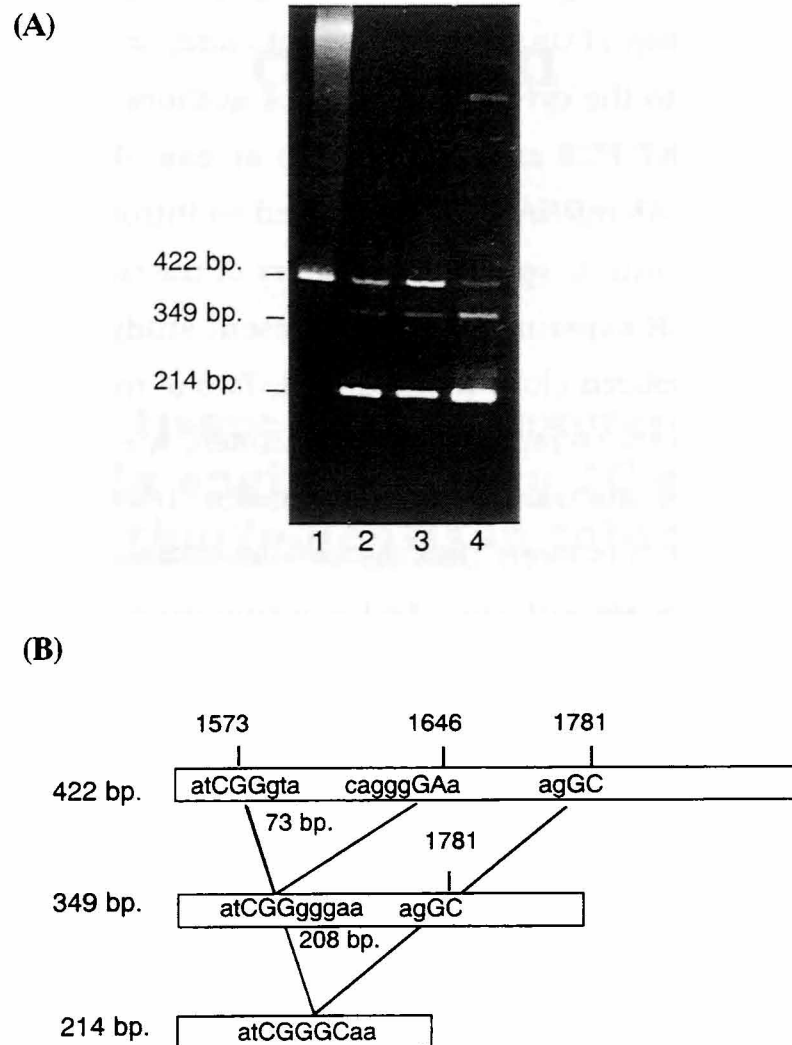


Fig. 2-5. RT-PCR of polyadenylated RNA from the tobacco plants transformed with plasmids pBTTW (BTW) and pBTTM (BTM).

(A): 5% TBE PAGE separation of the amplified products.

(B): Schematic diagram of cryptic splicing found in the cry 1Ab transcripts.

Lane 1, plasmid DNA (positive control); lane 2, BTM18; lane 3, BTW14; lane 4, BTW2.

Different mechanisms have been proposed to explain the low steady state levels of the wild type Bt mRNA in transgenic plants but none has been readily proven. One of the proposed mechanisms for the low cytoplasmic *cry* transcript levels is involving splicing at cryptic sites, leading to generation of unstable transcripts and/or poor transport from the nucleus to the cytoplasm [29]. The authors of this hypothesis demonstrated by RT-PCR experiments that at least three regions in the bacterial *cry 1Ab* mRNA were recognized as introns and were spliced out by the intron splicing machinery of the transgenic tobacco plants. The RT-PCR experiments in the present study also revealed *cry 1Ab* transcripts, spliced close to the 3' end. Two of the splicing sites were equivalent to those reported by van Aarsen, R et al. [29], however additional acceptor site was detected at position 1646 relative to translational start. It is likely that the smaller 73 bp. intron was spliced simultaneously with the 214 bp. intron from the same donor site but with lower efficiency (Fig. 2-5). Interestingly search of the truncated bacterial *cry 1Ab* gene for potential *Arabidopsis* splice sites with the NetPlantGene server:

(<http://www.cbs.dtu.dk/NetPlantGene.html>) [64] predicted all of the reported by van Aarsen, R et al. [29] splicing sites, however the acceptor site at position 1646 found in the present study was not predicted by this analyses. This result demonstrate that precise detection and removal of processing signals can be difficult due to their imprecise nature and supports the idea that a full reconstruction is a better and time saving strategy for optimizing the expression levels of Bt genes as compared to the partial, stepwise modification of the suspected deleterious regions of the gene.

## **Chapter III**

**Green tissue specific expression of  
genetically engineered cry 1C genes from  
Bacillus thuringiensis in tobacco plants**



### 3.1 INTRODUCTION

*Spodoptera litura* (tobacco leafworm) is a generalist caterpillar which feeds primary on the leaf tissues of the affected crop plants.. Although the transgenic crops expressing the synthetic *cry 1A*, *cry 3A* and *cry 2B* genes are already on the market [65], the relative resistance of *Spodoptera* species to Cry 1A type toxins and their sensitivity to only Cry 1C and Cry 9C toxins [49] prevents the utilization of the commercially available transgenic crop plants for control of *S. litura*.

The Cry 1C toxins from *B. thuringiensis* are 20-40 fold more toxic than Cry 1A type toxins towards *Spodoptera* [49]. Specific expression of the Cry 1C toxins into the leaf tissues of the transgenic crops may provide a useful approach to control this rather resistant insect species. The present study attempted to confer resistance towards *Spodoptera litura* larvae in transgenic tobacco plants by green tissue specific expression of the wild type and a reconstructed version of truncated *cry 1C* gene from *B. thuringiensis*.

Up to 200 fold difference in transgene expression levels were reported in transgenic plants upon the site of integration into the plant genome due to a phenomenon called “position effect” [66]. Therefore, search for appropriate markers which will allow for selection of the rare events of integration in favor of high expression levels has always been priority research in plant biotechnology. Improved expression levels and insecticidal effects were reported for truncated wild type *cry 1Ab* gene fused at the 3’ end to an *NPT II* gene [20]. The fusion allowed more stringent *in vitro* selection for the high expression levels of the fused protein. Coexpression of the *pat* gene with a synthetic *cry 1C* gene was also proposed to facilitate *in vitro* selection and breeding transfer of the linked *cry 1C* gene [44]. Another possible selection marker which will allow *in vitro* as well as field selection of tobacco plants transformed with the wild type *cry 1C* was tested in the present study. A

translational fusion of rat CYP 1A1 and yeast NADPH-P450 oxidoreductase (CYP1A1/YR) was shown to confer resistance to the herbicide chlorotoluron [52]. The *CYP1A1/YR* gene was cotransformed with the *cry 1C* gene in transgenic tobacco plants. The regenerated transgenic plants, selected *in vitro* for resistance to the herbicide chlorotoluron, were examined for insecticidal effects towards *S. litura* larvae.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Design of the synthetic *cry 1C* gene coding sequence**

The wild type *cry 1C* gene corresponding to the *cry 1Ca5* gene (Accession number X96682) and reported to encode for a protein representing the consensus of the known Cry 1C proteins was obtained from Dr. T. Yamamoto (Sandoz Agro, Inc., Palo Alto, CA, USA). The codon usage of the truncated wild type *cry 1C* gene coding for the amino acid residues from 1 to 655 was compared to the codon usage of the dicotyledonous *rbcS* gene sequences published. The codons which were abundant in the native *cry 1C* gene but were rarely used in dicotyledonous plant *rbcS* genes, were targeted for replacement (Table 3-1). The targeted codons were replaced at random and care was taken only for relatively even distribution of the codons throughout the sequence. The replacements made in the codon usage of the gene did not alter the amino acid sequence of the truncated Cry 1C protein (Fig. 3-1).

The reconstructed gene sequence was divided into five blocks separated by Sph I, Xho II, Pst I, Bgl II, Xba I and Sac I, respectively (Fig. 3-1). DNA segments were each designed as one antisense and three to five sense oligonucleotides ranging in length from 37 to 110 nt. with short overlaps of 12 to 14 bp ( Fig. 3-1). The oligonucleotides, produced by Kurabo Co. Ltd. (Osaka, Japan) as dried crude preparations were dissolved in a NANO® pure water to a concentration of 20 pM and were used in PCR reactions without any further purification steps. DNA segments were assembled by a modified PCR method in which mixtures of one antisense oligonucleotide and two to four overlapping sense oligonucleotides were amplified in a single PCR reaction.

Table 3-1 Codon Usage in a Truncated wild type *cry 1C* Gene Compared to that of 4 Dicotyledonous. RuBisCo Small Subunit Genes

|          | TR1CORF<br>(Truncated wt<br><i>cry 1Cas</i> ) |        | NSRUB1<br>(tobacco)<br>X01722 |        | TOMRBCSE<br>(tomato)<br>M15236 |        | POTRBCS<br>(potato)<br>J03613 |        | GMU39567<br>(soybean)<br>U39567 |        |
|----------|-----------------------------------------------|--------|-------------------------------|--------|--------------------------------|--------|-------------------------------|--------|---------------------------------|--------|
| codon-AA | Nr                                            | %      | Nr                            | %      | Nr                             | %      | Nr                            | %      | Nr                              | %      |
| TTT-Phe  | 39                                            | (5.95) | 1                             | (0.55) | 3                              | (1.66) | 2                             | (1.10) | 2                               | (1.12) |
| TTC-Phe  | 2                                             | (0.30) | 6                             | (3.31) | 4                              | (2.21) | 5                             | (2.75) | 8                               | (4.47) |
| TTA-Leu  | 32                                            | (4.88) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| TTG-Leu  | 4                                             | (0.61) | 5                             | (2.76) | 6                              | (3.31) | 7                             | (3.85) | 4                               | (2.23) |
| CTT-Leu  | 11                                            | (1.68) | 6                             | (3.31) | 4                              | (2.21) | 3                             | (1.65) | 5                               | (2.79) |
| CTC-Leu  | 0                                             | (0.00) | 1                             | (0.55) | 2                              | (1.10) | 2                             | (1.10) | 1                               | (0.56) |
| CTA-Leu  | 8                                             | (1.22) | 1                             | (0.55) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| CTG-Leu  | 4                                             | (0.61) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 2                               | (1.12) |
| ATT-Ile  | 26                                            | (3.96) | 3                             | (1.66) | 5                              | (2.76) | 5                             | (2.75) | 3                               | (1.68) |
| ATC-Ile  | 4                                             | (0.61) | 5                             | (2.76) | 3                              | (1.66) | 3                             | (1.65) | 6                               | (3.35) |
| ATA-Ile  | 21                                            | (3.20) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| ATG-Met  | 4                                             | (0.61) | 5                             | (2.76) | 6                              | (3.31) | 6                             | (3.30) | 7                               | (3.91) |
| GTT-Val  | 13                                            | (1.98) | 7                             | (3.87) | 7                              | (3.87) | 9                             | (4.95) | 4                               | (2.23) |
| GTC-Val  | 3                                             | (0.46) | 1                             | (0.55) | 3                              | (1.66) | 2                             | (1.10) | 1                               | (0.56) |
| GTA-Val  | 18                                            | (2.74) | 1                             | (0.55) | 1                              | (0.55) | 0                             | (0.00) | 1                               | (0.56) |
| GTG-Val  | 6                                             | (0.91) | 5                             | (2.76) | 4                              | (2.21) | 4                             | (2.20) | 5                               | (2.79) |
| TCT-Ser  | 19                                            | (2.90) | 1                             | (0.55) | 4                              | (2.21) | 1                             | (0.55) | 2                               | (1.12) |
| TCC-Ser  | 5                                             | (0.76) | 3                             | (1.66) | 2                              | (1.10) | 5                             | (2.75) | 5                               | (2.79) |
| TCA-Ser  | 7                                             | (1.07) | 6                             | (3.31) | 4                              | (2.21) | 4                             | (2.20) | 2                               | (1.12) |
| TCG-Ser  | 1                                             | (0.15) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| CCT-Pro  | 19                                            | (2.90) | 5                             | (2.76) | 5                              | (2.76) | 3                             | (1.65) | 2                               | (1.12) |
| CCC-Pro  | 1                                             | (0.15) | 0                             | (0.00) | 0                              | (0.00) | 2                             | (1.10) | 3                               | (1.68) |
| CCA-Pro  | 13                                            | (1.98) | 5                             | (2.76) | 5                              | (2.76) | 5                             | (2.75) | 7                               | (3.91) |
| CCG-Pro  | 4                                             | (0.61) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| ACT-Thr  | 12                                            | (1.83) | 5                             | (2.76) | 5                              | (2.76) | 4                             | (2.20) | 5                               | (2.79) |
| ACC-Thr  | 7                                             | (1.07) | 3                             | (1.66) | 2                              | (1.10) | 4                             | (2.20) | 5                               | (2.79) |
| ACA-Thr  | 19                                            | (2.90) | 0                             | (0.00) | 4                              | (2.21) | 1                             | (0.55) | 0                               | (0.00) |
| ACG-Thr  | 9                                             | (1.37) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| GCT-Ala  | 12                                            | (1.83) | 5                             | (2.76) | 6                              | (3.31) | 6                             | (3.30) | 7                               | (3.91) |
| GCC-Ala  | 3                                             | (0.46) | 6                             | (3.31) | 3                              | (1.66) | 5                             | (2.75) | 3                               | (1.68) |
| GCA-Ala  | 14                                            | (2.13) | 4                             | (2.21) | 5                              | (2.76) | 2                             | (1.10) | 3                               | (1.68) |
| GCG-Ala  | 4                                             | (0.61) | 1                             | (0.55) | 0                              | (0.00) | 2                             | (1.10) | 0                               | (0.00) |
| TAT-Tyr  | 17                                            | (2.59) | 2                             | (1.10) | 1                              | (0.55) | 0                             | (0.00) | 0                               | (0.00) |
| TAC-Tyr  | 3                                             | (0.46) | 8                             | (4.42) | 9                              | (4.97) | 10                            | (5.49) | 8                               | (4.47) |
| TAA-***  | 1                                             | (0.15) | 1                             | (0.55) | 1                              | (0.55) | 1                             | (0.55) | 1                               | (0.56) |
| TAG-***  | 0                                             | (0.00) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| CAT-His  | 9                                             | (1.37) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| CAC-His  | 2                                             | (0.30) | 1                             | (0.55) | 1                              | (0.55) | 2                             | (1.10) | 2                               | (1.12) |
| CAA-Gln  | 18                                            | (2.74) | 6                             | (3.31) | 7                              | (3.87) | 4                             | (2.20) | 4                               | (2.23) |
| CAG-Gln  | 8                                             | (1.22) | 4                             | (2.21) | 2                              | (1.10) | 4                             | (2.20) | 3                               | (1.68) |
| AAT-Asn  | 43                                            | (6.55) | 2                             | (1.10) | 4                              | (2.21) | 3                             | (1.65) | 1                               | (0.56) |
| AAC-Asn  | 8                                             | (1.22) | 7                             | (3.87) | 4                              | (2.21) | 7                             | (3.85) | 6                               | (3.35) |
| AAA-Lys  | 6                                             | (0.91) | 2                             | (1.10) | 3                              | (1.66) | 3                             | (1.65) | 0                               | (0.00) |
| AAG-Lys  | 1                                             | (0.15) | 9                             | (4.97) | 8                              | (4.42) | 7                             | (3.85) | 11                              | (6.15) |
| GAT-Asp  | 28                                            | (4.27) | 3                             | (1.66) | 3                              | (1.66) | 2                             | (1.10) | 4                               | (2.23) |
| GAC-Asp  | 4                                             | (0.61) | 2                             | (1.10) | 3                              | (1.66) | 4                             | (2.20) | 3                               | (1.68) |
| GAA-Glu  | 29                                            | (4.42) | 5                             | (2.76) | 3                              | (1.66) | 3                             | (1.65) | 3                               | (1.68) |
| GAG-Glu  | 7                                             | (1.07) | 7                             | (3.87) | 8                              | (4.42) | 8                             | (4.40) | 6                               | (3.35) |
| TGT-Cys  | 3                                             | (0.46) | 0                             | (0.00) | 1                              | (0.55) | 0                             | (0.00) | 0                               | (0.00) |
| TGC-Cys  | 2                                             | (0.30) | 4                             | (2.21) | 3                              | (1.66) | 4                             | (2.20) | 4                               | (2.23) |
| TGA-***  | 0                                             | (0.00) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| TGG-Trp  | 9                                             | (1.37) | 5                             | (2.76) | 5                              | (2.76) | 5                             | (2.75) | 4                               | (2.23) |
| CGT-Arg  | 6                                             | (0.91) | 3                             | (1.66) | 2                              | (1.10) | 5                             | (2.75) | 3                               | (1.68) |
| CGC-Arg  | 4                                             | (0.61) | 1                             | (0.55) | 1                              | (0.55) | 0                             | (0.00) | 2                               | (1.12) |
| CGA-Arg  | 7                                             | (1.07) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| CGG-Arg  | 3                                             | (0.46) | 0                             | (0.00) | 0                              | (0.00) | 0                             | (0.00) | 0                               | (0.00) |
| AGT-Ser  | 10                                            | (1.52) | 2                             | (1.10) | 2                              | (1.10) | 1                             | (0.55) | 0                               | (0.00) |
| AGC-Ser  | 5                                             | (0.76) | 3                             | (1.66) | 4                              | (2.21) | 4                             | (2.20) | 2                               | (1.12) |
| AGA-Arg  | 21                                            | (3.20) | 2                             | (1.10) | 2                              | (1.10) | 2                             | (1.10) | 1                               | (0.56) |
| AGG-Arg  | 8                                             | (1.22) | 1                             | (0.55) | 1                              | (0.55) | 0                             | (0.00) | 3                               | (1.68) |
| GGT-Gly  | 12                                            | (1.83) | 0                             | (0.00) | 2                              | (1.10) | 2                             | (1.10) | 4                               | (2.23) |
| GGC-Gly  | 3                                             | (0.46) | 4                             | (2.21) | 1                              | (0.55) | 0                             | (0.00) | 6                               | (3.35) |
| GGA-Gly  | 28                                            | (4.27) | 6                             | (3.31) | 6                              | (3.31) | 8                             | (4.40) | 5                               | (2.79) |
| GGG-Gly  | 7                                             | (1.07) | 0                             | (0.00) | 1                              | (0.55) | 1                             | (0.55) | 0                               | (0.00) |

In brief, the PCR cocktail (100 µl) contained 1x rTaq® or Taq Plus Precise® polymerase buffer, 0.25 mM of each dNTPs, 20U rTaq® (Takara, Kyoto, Japan) or Taq Plus Precise® polymerase mix (Stratagene, CA, USA), 20 pM of each antisense and the most distal sense oligonucleotide and 0.2 pM of the internal overlapping primers. Twenty to thirty cycles were performed at 95 °C for 30 sec., 50 °C for 30 sec. and 72 °C for 30 sec. Five microliters of the PCR reaction mixture were added to the second PCR mixture containing the same antisense primer, combined with the oligonucleotides, distal to the amplified fragment and the second PCR reaction was carried out in the same conditions as the first PCR. In the cases when non-specific priming caused a problem the desired fragment was purified by PAGE as described [55] before the second PCR. Each full length block was PAGE purified and cloned in pT7Blue T-vector (Novagene, CA, USA) for sequencing. Blocks 1 and 2, and blocks 3 and 4 (Fig. 3-1) were reamplified by PCR for 10 cycles with the M13 universal primers and Pfu® polymerase (Stratagene, CA, USA), digested with XhoII or BglII, respectively, and ligated with T4 DNA ligase after PAGE purification. The ligated products were reamplified for additional 20 cycles with the universal primers and Pfu polymerase (Stratagene) and cloned into pUC19 as SphI/PstI or PstI/XbaI fragments, respectively (Fig. 3-1). The sequences of each block were verified by double stranded sequencing on a Hitachi model 550L Automated DNA sequencer according to the manufacturers instructions. Finally, the full length sequence of the modified truncated *cry 1C* gene coding for the amino acid residues 1-655, was assembled in a pUC19 plasmid named pUSynC1. The junctions resulted from the ligation of each block were each verified by sequencing.



### 3.2.2 Construction of recombinant plasmids

The plasmid pRBT containing the promoter region and the chloroplast targeting signal of *rbcS* gene of *Nicotiana plumbaginifolia* was provided by Dr. N. H. Chua (The Rockefeller University, NY) and the plasmid pSB629 containing the wild type *cry 1C* gene was obtained from Dr. T. Yamamoto (Sandoz Agro, Ltd, CA, USA). The recombinant DNA procedures were carried out according to Sambrook et al. [55] unless otherwise was mentioned.

A Xho I linker was inserted into the Hind III site at the 5' of the *rbcS* promoter in the plasmid pRBT. Insertion of a synthetic adapter containing Sph I and Nco I sites allowed *in frame* fusion of the 3.5 kb Nco I /Eco RI fragment of the plasmid pSB629 containing the *cry 1C* gene to the transit peptide of *rbcS* gene of *N. plumbaginifolia* [67] to yield the plasmid pURBCF. A Bam HI /Eco RI fragment from the plasmid pBUR1 [68] containing the *nos* polyadenylation sequences and a TAA stop codon was inserted into Eco RI site and the Bcl I site at position 1960 relative to the translational start of the wild type *cry 1C* gene of the plasmid pURBCF. The resulted plasmid pUR1CNT contained a part of the *cry 1C* gene coding for the amino acids 1-655 including the active fragment which was fused *in frame* to the *rbcS* transit peptide and the TAA stop codon. The wild type *cry 1C* gene in the plasmid pUR1CNT was replaced for the Nco I/Sac I fragment of the plasmid pUSynC1 containing the synthetic gene coding sequence. The resulting plasmid pURSC1 had the synthetic *cry 1C* gene between the *rbcS* promoter and *nos* polyadenylation sites (Fig. 3-2).

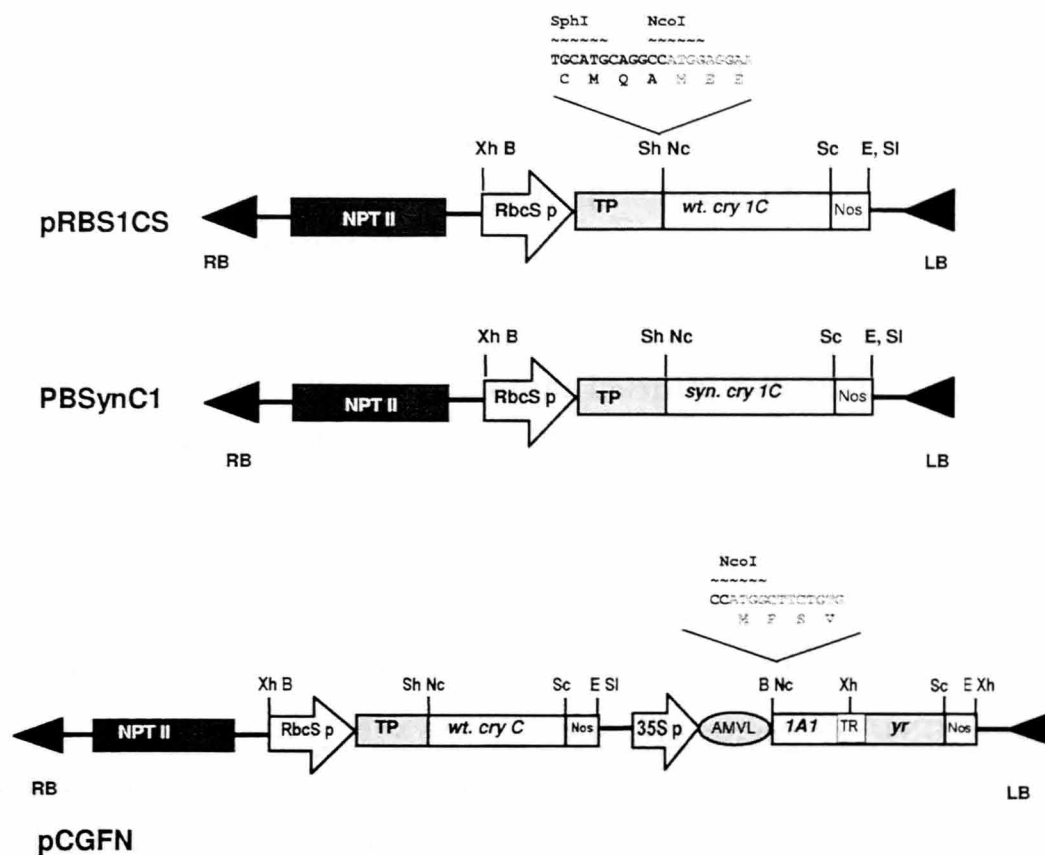


Fig. 3-2. Schematic representation of the recombinant expression plasmids pRBS1CS and pBSynC1 for the expression of the wild type and synthetic *cry 1C* genes, and the plasmid pCGFN for the coexpression of both the wild type *cry 1C* gene and *rat CYP1A1/yeast P450 oxidoreductase* fusion gene.

Nos, *nos* 3' UTR; LB, left T-DNA border; RB, right T-DNA border; 35Sp, CaMV 35S promoter; RbcSp, *N. plumbaginifolia* RuBisCo small subunit (*rbcS*) promoter; TP, RbcS transit peptide; CYP1A1, rat CYP1A1; yr, yeast NADPH-cytochrome P450 oxidoreductase; *wt cry 1C*, truncated wild type *cry 1C* gene coding for amino acids 1-655; *syn cry 1C*, synthetic *cry 1C* gene shown on Fig. 1; B, Bam HI; E, Eco RI; H, Hind III; Nc, Nco I; Sc, Sac I; Sh, Sph I; Sl, Sal I, Xh, Xho I

Preservation of the open reading frame was confirmed by sequencing of the junctions. Finally the 3.5 kb. *cry 1C* expression cassettes for the wild type and the synthetic *cry 1C* genes from the plasmids pUR1CNT and pURSC1 were each inserted as a Xho I/ Eco RI fragments into a pBI121 derivative in which CaMV 35S promoter was replaced by a Xho I linker to yield the plasmids pRBS1CS and pBSynC1, respectively (Fig. 3-2). The plasmid pCGFN was constructed as Nco I and Bam HI sites were introduced by PCR at the translational start of rat *CYP 1A1* in the plasmid pGFC1 [52], then a 3.5 kb. Bam HI/Sac I fragment containing *rat CYP 1A1/yeast P450 oxidoreductase* fusion was inserted into pBUR1 [68] and finally an Sal I/Apa I fragment from pRBS1CS containing the *rbcS/cry 1C* cassette was inserted to yield the plasmid pCGFN (Fig. 3-2). The sequences in all PCR mutagenesis steps were verified by dideoxy sequencing on a Hitachi model 550L automatic sequencer by the fluorescent dye primer method according to the manufacturers instructions.

### **3.2.3 Tobacco transformation**

Haploid tobacco (*Nicotiana tabacum*) cv. Samson nn., originated from microspore culture and maintained *in vitro* was used for transformation. *Agrobacterium* leaf disc transformation by using the *Agrobacterium tumefaciens* strain LBA 4404 was done essentially as described [58]. Regenerated haploid shoots were selected on a medium containing 100 mg/l kanamycin and propagated *in vitro*.

### **3.2.4 NPT II ELISA**

Total protein was extracted from 0.1 g. of leaf tissue in the extraction buffer ( 100 mM Tris, 50 mM EDTA and 10 mM PMSF (pH 7.5). Protein concentration was determined by the use of a BioRad® protein assay kit with IgG as a standard and diluted to 400mg/l with the same buffer. ELISA was performed by the use of a NPT II ELISA kit

(5 Prime-3 Prime, Boulder, CO, USA) with a 25X dilution of the 400 mg/l samples according to the manufacturers instructions and plates were scanned at  $\lambda=405$  nm after 15 minutes color development

### **3.2.5 Genomic PCR**

Genomic DNA was extracted from 0.1 g of leaf tissues by the method of Delaporta [59]. PCR was performed with an AmpliTaq Gold<sup>®</sup> in 50  $\mu$ l reactions 1X buffer 0.2 mM of each dNTPs, AmpliTaq Gold<sup>®</sup> enzyme, 50 pM of each forward 5'-CAGGATTTACAGGAGGGGAT-3' (1CF) and reverse 5'-GATAATCCGTCACATCGGT-3' (1CR) primers, which amplify 492 bp fragment of *cry 1C* gene, and 0.5 mg genomic DNA. The reaction mixture for the multiple PCR experiments contained primers 5'-AACCTACCTGTTCACTACGA-3' (S3) and 5'-TTACCAGACATCTTCTTCCT-3'(S4) , amplifying 595 bp fragment of YR, in addition to primers 1CF and 1CR, described above. Amplification was done for 35 cycles at 95 °C 45 sec, 55 °C 1 min. and 72 °C 2 min and 5  $\mu$ l of the reactions were analyzed by electrophoresis on 1% or 2% TAE agarose gel.

### **3.2.6 RT-PCR experiments**

Polyadenylated RNA was isolated from leaf tissues by the use of a Micro mRNA purification kit (Pharmacia). The first strand cDNA was synthesized from 200 ng poly A<sup>+</sup> RNA by the use of the first strand cDNA synthesis kit (Pharmacia) with the provided oligo dT 18 bifunctional primer and following the manufacturers instructions. Five  $\mu$ l of the first strand reactions were amplified by 3'-RACE PCR with the primers forward 1CF and reverse 5'-AACTGGAAGAATTCGCGGCCGC-3' (bifunctional primer anchor). PCR was carried out with a rTaq<sup>®</sup> (Takara) in the provided buffer for 30 cycles at 95°C for 30 sec., 55°C for 30 sec

and 72°C for 1 min. Five µl of the 3'-RACE PCR reactions were reamplified for additional 30 cycles with the same forward primer 1CF and a nested reverse primer 1CR giving amplified fragment of 492 bp. Ten µl of the reactions were analyzed for electrophoresis.

### **3.2.7 Northern blot**

Polyadenylated RNA was extracted from leaf tissues of transgenic plants by the use of a Micro mRNA purification kit (Pharmacia, Upsala, Sweden) as described by the vendor. One µg of mRNA was subjected to formamide agarose electrophoresis and transferred to a Hybond N<sup>+</sup> membrane (Amersham, London, UK) by a semidry capillary blot method [55] by using 10X SSC. Hybridization was carried out at 42°C overnight in a hybridization buffer containing 50 % formamide with probes, generated by PCR with the above described primers and specific to the 3' end of the truncated *cry 1C* or *yeast P450 oxidoreductase* genes, which were labeled with [ $\gamma$ -<sup>32</sup>P] dCTP by the random primer method. The 810 bp DNA fragment at the 5' end of the synthetic *cry 1C* gene was generated by PCR with the primers : forward: 5'-GAGGTAGGATCCATGGAGGAAAACAATCAGAACC-3' and reverse: 5'-GAGGTAGGATCCATGGAGGAAAACAATCAGAACC-3' . The fragment was labeled by random primer method as described above and used as a probe for detection of the synthetic *cry 1C* gene mRNA. The blots were washed at high stringency (0.2 x SSC, 0.1% SDS at 65 °C) according to manufacturers instructions (Amersham, London, UK).

### **3.2.8 Protein extraction and Western blot**

A crude leaf protein fraction was extracted from 0.2 g of grinded leaf tissues with 400 µl 0.1 N Na OH for 30 min on ice as described [69]. Extracts were neutralized with 80 µl of 1 N Tris/HCl, pH=4.5 and centrifuged in a microfuge for 10 minutes. Protein concentration in the

supernatant was measured by a BioRad protein assay with BSA as a standard. Microsome fractions from tobacco tissues were prepared essentially according to Shiota et al. [52]. Twenty  $\mu\text{g}$  of crude protein extracts or 50  $\mu\text{g}$  of microsomal fractions were each subjected to 8% SDS-PAGE and transferred to a PVDF membrane (MSI, MA, USA). Rabbit antiserum was raised against inclusion bodies prepared from *E. coli* cells carrying a truncated native *cry 1C* gene coding for the N-terminal 645 amino acids in a pET32a expression vector. The Cry 1C protein was detected with 1:1000 dilution of rabbit antiserum followed by alkaline phosphatase conjugated Goat Anti Rabbit antibodies and visualized by staining in BCIP and NBT at pH 9.5. Levels of Cry 1C protein were quantified from scanned blots in a MacBas<sup>®</sup> Imaging Software (Fuji Film, Tokyo, Japan) and compared to the lanes containing known amounts of SDS-PAGE purified *E. coli* cells expressed active fragment of Cry 1C protein. The rat CYP1A1/yeast P450 oxidoreductase fusion protein was detected with a Goat Anti-Rat CYP1A1 antibody followed by alkaline phosphatase labeled Rabbit Anti Goat antibody and visualized by staining in BCIP and NBT at pH 9.5. The background hybridization was taken for a base and the relative levels of rat CYP1A1/yeast P450 oxidoreductase protein as were quantified from the scanned blot by using MacBas<sup>®</sup> Imaging Software (Fuji Film, Tokyo, Japan) and expressed in arbitrary units.

### **3.2.9 Chlorotoluron Selection of transgenic plants**

Transgenic plants rooted on medium containing 100 mg/l kanamycin (Km) were cut into two. The upper part was transferred on a Km containing medium and the lower (rooted) part was transferred to a MS medium containing 50  $\mu\text{M}$  of the herbicide chlorotoluron. The results were recorded after 30 days of incubation.

### **3.2.10 Bioassay with *S. litura* Larvae**

Five freshly hatched larvae of *S. litura* were infested on each detached tobacco leaf from *in vitro* grown haploid tobacco plants transformed with the plasmids pRBS1CS or pCGFN in three replications in petri dishes overlaid with damped filter paper and incubated at 25°C under 16/8h light/dark cycle 15, 000 lux light conditions. In the case of pBSynC1 transformed tobacco plants ten larvae were infested on leaf tissues from plants, grown in pots with soil and irrigated with 1000 fold diluted Hyponex<sup>®</sup>, in three replications. Tests were otherwise done in the same way as described above.

At the end of the experiment (three or six days) mortality was recorded and weights of individual survived insect larvae were measured on a electronic microbalance.

### **3.2.11 Production of dihaploids and germination tests**

The greenhouse grown tobacco plants were decapitated at the time of flowering and the lateral buds were treated with 0.1% w/w colchicine in lanolin. The plants which were able to produce seeds were considered diploid.

The T<sub>1</sub> seeds were surface sterilized in 10% kitchen bleach containing 0.1% Tween 20 for 3 minutes and extensively washed in sterile distilled water before plating on petri dishes containing 1X MS medium (pH 5.7), 30 g/l sucrose 8 g/l agar and 100 g/l kanamycin. The results for kanamycin resistance were scored after 2 weeks of incubation 26 °C 16/8 light/dark cycle.

### **3.3 RESULTS**

#### **3.3.1 Construction of the synthetic *cry 1C* gene**

Comparison of the codon usage of the truncated wild type *cry 1C* gene and the published dicot *rbcS* gene sequences [54] identified the rare codons in the coding sequence of *cry 1C* gene. The replacement of 288 codons in the wild type *cry 1C* biased the codon usage of the gene to conform that of the *rbcS* genes. The GC content in the redesigned gene was increased from 36.6% in the wild type gene to 45.1% in the synthetic gene (Fig. 3-1) although, the amino acid sequence of the gene was not affected by the replacement of the codons. Interestingly the codon replacements automatically removed some motifs, known to interfere with the mRNA stability, from the coding sequence. These included 22 potential polyadenylation sites [70], 11 ATTTA motifs [31] and 23 stretches of more than four consecutive T bases[71].

The redesigned gene was assembled from crude oligonucleotides ranging in length from 37 nt. to 110 nt. by a modified PCR method (Fig 3-1.). The overlapping parts of the megaprimers were selected on the base of the common rules for design of PCR primers and the hairpin structures in the overlaps were strongly avoided. In general 12 to 14 nucleotide overlaps with estimated  $T_m$  40-42 °C were sufficient for annealing even at 50 °C. PCR amplification of a mixture of 3 to 5 megaprimers allowed to minimize the total number of cycles required for the syntheses of the full length blocks and the specific design with one antisense and several sense primers minimized the number of overlapping bases. In general 40 to 60 cycles were sufficient to produce the full length of the desired block. In addition, the method was faster and less labor intensive than the classical designs of PCR based gene syntheses [35]. We tested several thermostable DNA polymerases including a Vent® (Epicentre Technologies, WI, USA), Pfu® and TaqPlus® (Stratagene, CA, USA) and Expand™ from Boeringer Mainhaim

(Mainhaim, Germany). Unfortunately, the Vent® and Pfu® polymerases, which have a proof-reading capability and the lowest error rates, did not work for the purpose. The best results were obtained by the use of TaqPlus® Precise PCR system from Stratagene, which is a mixture of Taq® and Pfu® polymerases and the rTaq® (Takara, Kyoto, Japan). However, the recovery rate of the desired sequences appeared to be independent on the polymerase used. Five to eight clones of each PCR synthesized block cloned in pT7Blue T-vector were sequenced to select the clones containing the desired sequence. The mean recovery rate for all blocks was 25%. However, it was variable for each block. Interestingly the highest recovery of 43% (3 of 7 clones) was observed for the block 5 (406 bp.) (Fig. 3-1) which was assembled in two rounds of PCR in the total of 56 cycles with rTaq® from Takara. Surprisingly, the lowest recovery rate 12.5% (1 of 8 clones) was found for block 4 (381 bp.) which was the shortest block consisting of only 4 synthetic oligonucleotides and was assembled in the total of 40 cycles with the Expand™ polymerase which have the proof-reading capability. Most of the observed mutations occurred as small insertions or deletions, which were located in the overlapping regions of the synthetic oligonucleotides. Therefore, we concluded that the mutations were originating from the crude oligonucleotide preparations rather than introduced by the thermostable polymerases. This view was also confirmed with the results from the reamplification of the ligated blocks 1 and 2 and 3 and 4, respectively. The reamplification was done with a proof-reading Pfu® polymerase. The observed mutations in these cases also were located at the 5' and 3' ends of the amplified fragments covered by the megaprimer.

### **3.3.2 Expression of the truncated wild type *cry 1C* gene in haploid tobacco plants**

The truncated wild type *cry 1C* gene coding for the N-terminal 655 amino acids of the protoxin was fused to the *rbcS* promoter from *N. plumbaginifolia* which is a strong green tissue specific promoter. The *rbcS* untranslated leader and the transit peptide which were shown to improve transgene expression levels directed from *rbcS* promoters [36] were also included. The gene coding sequence was fused in frame to the RbcS transit peptide by a synthetic linker which added two amino acids (QA) (Fig. 3-2). Thirty seven haploid shoots transformed with the plasmid pRBS1CS containing the wild type *cry 1C* gene under the control of the green tissue specific tobacco *rbcS* promoter were selected for kanamycin resistance. The regenerated shoots resistant to 100 µg/ml kanamycin, were tested for the expression levels of the cotransformed NPT II protein by a quantitative ELISA method. The results of the selection of the tobacco plants, transformed with the plasmid pRBS1CS are summarized in Table 3-2. Nine of the regenerated plants showed undetectable NPT II levels. The NPT II levels for 7 of the plants were between 0.5 and 5 ng. per mg. leaf protein, 8 plants showed levels 5-10 ng/mg, 9 were between 10 and 15 ng./mg and 4 showed more than 15 ng NPT II per mg leaf protein. Ten plants, which showed levels above 11 ng NPT II per mg protein, were selected for further analyses. The stable integration of the *cry 1C* gene and the presence of the *cry 1C* transcript in all of the selected plants were confirmed by PCR and RT-PCR , respectively (Table 3-2). However, the Cry 1C protein was below the detection limit of the western blot (data not shown).

The insecticidal activity of selected plants transformed with pRBS1CS was tested towards neonate *Spodoptera litura* larvae in detached leaf bioassays. The individual survived larvae were weighted after six days of feeding. All of the selected transgenic plants inhibited development of larvae allowed to feed on the leaf tissues from them as compared to the control. The mean weight of the survived larvae ranged

from  $8.08 \pm 0.20$  to  $3.1 \pm 0.11$  mg. which compared to the weight of the control larvae ( $13.20 \pm 0.61$  mg) makes 39 to 77 % reduction(Fig. 3-3). Three of the plants RC26, RC17 and RC24 inhibited the larval growth by more than 70 %. The higher insecticidal effects of these plants correlated with the NPT II levels ( $r=-0.833$ ), suggesting that the higher insect resistance was due to the higher levels of the coexpressed Cry 1C protein in these plants.

Table 3-2 Summary of selection of pRBS1CS transformed tobacco plants expressing the truncated wt *cry 1C* gene.

| <i>Plant #</i> | <i>NPT II</i><br>(ng/mg) | <i>cry 1C</i><br><i>PCR</i> | <i>cry 1C</i><br><i>RT-PCR</i> |
|----------------|--------------------------|-----------------------------|--------------------------------|
| RC1            | 11.3                     | +                           | +                              |
| RC2            | >0.5                     | NA                          | NA                             |
| RC3            | 12.4                     | +                           | +                              |
| RC4            | 0.6                      | NA                          | NA                             |
| RC5            | 18.4                     | +                           | +                              |
| RC6            | 5.3                      | NA                          | NA                             |
| RC7            | 7.5                      | NA                          | NA                             |
| RC8            | 5.7                      | NA                          | NA                             |
| RC9            | 14.4                     | +                           | +                              |
| RC10           | 8.1                      | NA                          | NA                             |
| RC11           | 8.7                      | NA                          | NA                             |
| RC12           | 9.4                      | NA                          | NA                             |
| RC13           | 5.0                      | NA                          | NA                             |
| RC14           | 3.7                      | NA                          | NA                             |
| RC15           | >0.5                     | NA                          | NA                             |
| RC16           | 16.9                     | +                           | +                              |
| RC17           | 13.1                     | +                           | +                              |
| RC18           | 11.8                     | +                           | +                              |
| RC21           | 2.6                      | NA                          | NA                             |
| RC22           | >0.5                     | NA                          | NA                             |
| RC23           | >0.5                     | NA                          | NA                             |
| RC24           | 18.6                     | +                           | +                              |
| RC25           | 2.1                      | NA                          | NA                             |
| RC26           | 13.3                     | +                           | +                              |
| RC27           | 10.4                     | NA                          | NA                             |
| RC28           | 0.9                      | NA                          | NA                             |
| RC29           | >0.5                     | NA                          | NA                             |
| RC30           | >0.5                     | NA                          | NA                             |
| RC31           | 2.8                      | NA                          | NA                             |
| RC32           | >0.5                     | NA                          | NA                             |
| RC33           | >0.5                     | NA                          | NA                             |
| RC34           | 11.8                     | +                           | +                              |
| RC35           | 5.3                      | NA                          | NA                             |
| RC36           | 15.9                     | +                           | +                              |
| RC37           | >0.5                     | NA                          | NA                             |
| RC38           | 5.3                      | NA                          | NA                             |
| RC39           | 10.7                     | NA                          | NA                             |
| control        | 0.0                      | -                           | -                              |

NA-not attempted

\* mean weight of the survived larvae from two replications, five larvae each

\*\* standard deviation between replications

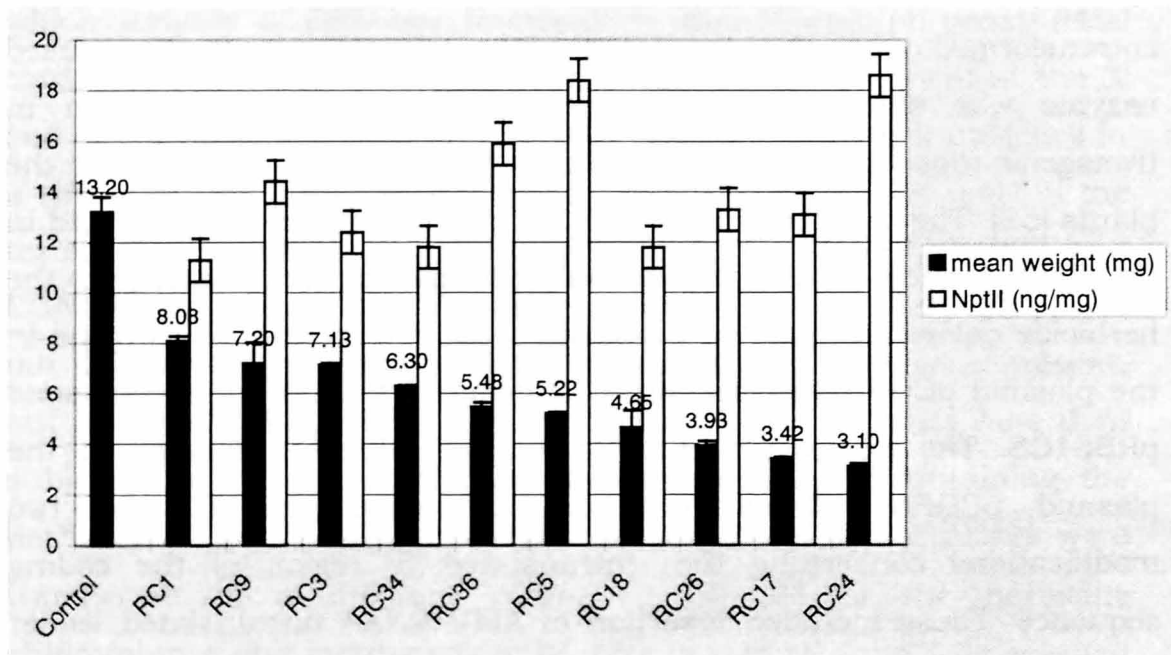


Fig. 3-3. NPT II protein levels and the insecticidal activities of transgenic tobacco plants expressing the wild type *cry 1C* gene under the control of *rbcS* promoter.

Solid bars represent the mean weight of the larvae in bioassay in mg. and the open bars show NPT II levels in ng. per mg. leaf protein. The control is untransformed haploid tobacco cv. *Samson nn*. The bioassay experiment was performed with the detached leaves from *in vitro* grown tobacco and neonate *S. litura* larvae. Five larvae were infested per leaf and incubated at 25° C, 16/8 light/dark cycle. The weights of each survived larvae was measured after six days. The error bars represent standard deviation between replications of the bioassay experiment and the standard error of NPT II protein measurement, respectively.

### **3.3.3 Coexpression of both truncated wild type *cry 1C* and *rat CYP1A1/yeast P450 oxidoreductase* genes in tobacco plants**

In an attempt to improve the selection for rare *cry 1C* gene integration events in favor of high expression levels a fusion gene between *rat CYP1A1* (cytochrome P450 monooxygenase) and *NADPH cytochrome P450 oxidoreductase* from yeast (*CYP1A1/YR*) was cotransformed with the *cry 1C* gene in tobacco plants. The fused enzyme was shown to metabolize the herbicide chlorotoluron in transgenic tobacco plants and to confer the herbicide resistance to the plants [52]. The coexpression of *CYP1A1/YR* was therefore attempted in the present study as an additional selection marker for resistance to the herbicide chlorotoluron. The expression cassette for the *cry 1C* gene in the plasmid pCGFN was the same as described above for the plasmid pRBS1CS. The expression cassette for the *CYP1A1/YR* gene in the plasmid pCGFN differed from the published one [52] by two modifications concerning the untranslated 5' region of the coding sequence. Those included insertion of AMV RNA4 untranslated leader [61] and conversion of the start codon context to the plant consensus sequence [72] by engineering of a Nco I site at the start codon (Fig. 3-2). To minimize the possibility of transgene silencing, the expression of *cry 1C* and *CYP1A1/YR* transgenes was directed from two different promoters *rbcS* and *CaMV 35S* which have different expression patterns. The *rbcS* promoter will direct the expression of *cry 1C* gene mainly in the leaf and green tissues, which is the main feeding target for *Spodoptera* larvae. Another reason to choose these promoters was that the gene products would be also targeted to different cell compartments. The Cry 1C protein should be sequestered to the chloroplasts by the RbcS transit peptide and *CYP1A1/YR* fusion protein should be lead to the microsomes by the native *CYP1A1* microsome targeting signal. So, the possibility of interference between the transgene protein products was theoretically minimized in this plasmid construct.

Forty-five tobacco plants resistant to 100 mg/l kanamycin were regenerated from the haploid Samson nn leaf disks transformed with the plasmid pCGFN. The kanamycin resistant transgenic tobacco plants were tested for the presence of *cry 1C* and *CYP1A1/YR* transgenes by a multiple PCR technique. Using four primers S3/S4 , which amplify a 585 bp. fragment at the 3' end of *yeast P450 oxidoreductase*, and 1CF/1CR, amplifying 492 bp. fragment at the 3' end of *cry 1C* gene (Fig. 3-4A), allowed detection of both transgenes in a single PCR reaction. Both transgenes were integrated in most of the tested plants with the exception of four CGN9, CGN10, CGN34 and CGN35, respectively which showed presence of the *cry 1C* gene, but not *CYP1A1/YR* and one (CGN1) that had *CYP1A1/YR* gene present, but not *cry 1C* (Fig. 3-4B). The kanamycin resistant plants were then subjected to a second selection step on a medium containing the herbicide chlorotoluron. The rooted kanamycin resistant plants were transferred to a medium containing 50  $\mu$ M of the herbicide chlorotoluron and incubated for 30 days in this medium. The selected concentration of the herbicide and incubation period was sufficient for the plants expressing low levels of CYP1A1/YR protein to bleach and dye (Fig. 3-4C). Although most of the plants were susceptible to the selection procedure, five plants (out of 45 tested) showed little or no damage by the herbicide (eg. CGN36 and CGN39, Fig. 3-4C). These plants, namely CGN19, CGN21, CGN22, CGN36 and CGN39 were selected for further characterization.

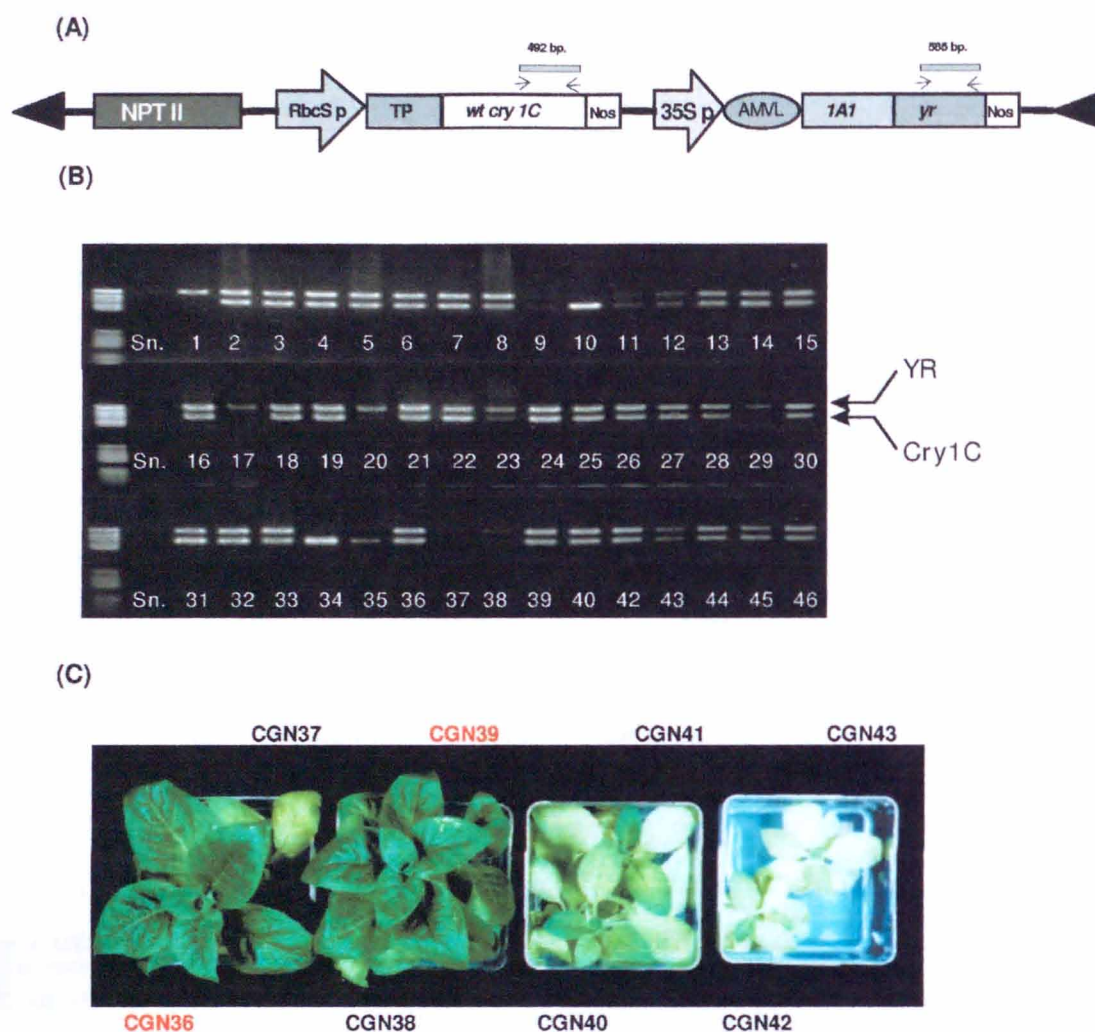


Fig. 3-4. Screening of kanamycin resistant tobacco plants coexpressing wild type *cry 1C* and *rat CYP1A1/yeast P450 oxidoreductase (CYP1A1/YR)* genes.

- (A): Schematic representation of the plasmid pCGFN depicting positions of the primers used for PCR.;
- (B): Confirmation of *cry 1C* and *CYP1A1/YR* genes integration into pCGFN transformed tobacco plant genomes by multiple PCR. The PCR products were separated in 2% TAE agarose. The numbers on the bottom correspond to the numbers of CGN plants independently transformed with the plasmid pCGFN. Sn, Samson nn. (control) ;M, marker.;
- (C): Selection of transgenic plants on MS medium, containing 50  $\mu$ M of the herbicide chlorotoluron. CGN36-CGN43, independent transformants coexpressing *cry 1C* and *CYP1A1/YR* genes.

RNA hybridization experiments showed that the *cry 1C* mRNA levels were still below the detection limit on the Northern blot of 2 µg poly A<sup>+</sup> RNA fraction (Fig 3-5A). Reprobing of the same blot with the probe specific to the 3' end of the *yeast P450 oxidoreductase* gene revealed a distinct hybridization signal of the expected size of 3.5 kb. corresponding to the full length of *CYP1A1/YR* transcript which was evident in all of the selected chlorotoluron resistant plants and was not present in the control (Fig. 3-5B). The more sensitive RT-PCR method confirmed that the *cry 1C* gene was transcribed (Fig. 3-5C). However, no conclusions about the intactness of the *cry 1C* transcript could be drawn from the available RT-PCR data. The presence of the *CYP1A1/YR* protein in the microsome fraction of the transgenic plants transformed with pCGFN was confirmed by western blot (Fig. 3-6A).

The chlorotoluron selected tobacco plants were also tested for the insecticidal effect towards neonate *S. litura* larvae following the protocol described above for the plants transformed with the plasmid pRBS1CS. The mean weights of the survived larvae was in the same range as observed for the plants transformed with the plasmid pRBS1CS (Fig. 3-6B). Although the Cry 1C protein was undetectable in the western blot (data not shown), comparison of the relative levels of *CYP1A1/YR* protein with the bioassay data showed strong negative correlation between *CYP1A1/YR* levels and the mean weight of the survived larvae ( $r=-0.945$ ) (Fig. 3-6B). These data provide an indirect evidence that the selection for chlorotoluron resistance isolated transgenic plants expressing the Cry 1C protein at higher levels and imply on the usefulness of the *CYP1A1/YR* as a distal selection marker in tobacco transformation.

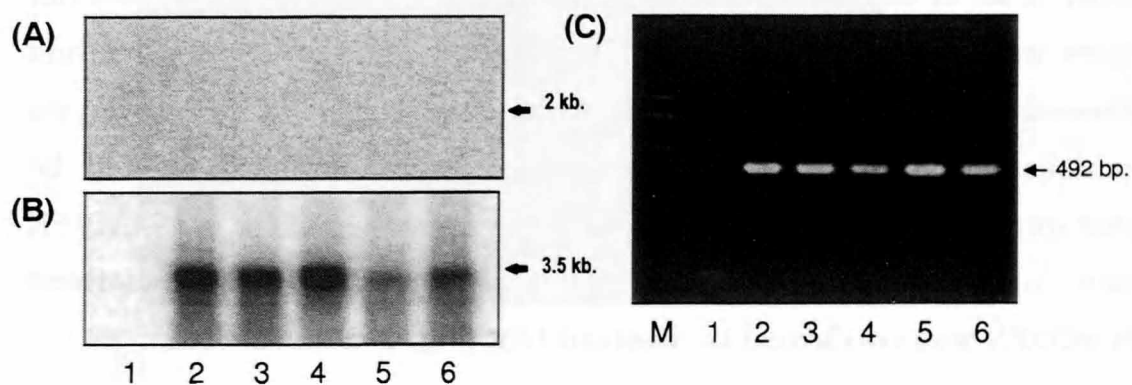


Fig. 3-5. Detection of *CYP1A1/YR* and *cry 1C* transcripts in tobacco plants coexpressing wild type *cry 1C* and *CYP1A1/yeast P450 oxidoreductase* genes. M, marker; 1, untransformed control; 2, CGN19; 3, CGN21; 4, CGN22; 5, CGN36; 6, CGN39.

- (A): Northern blot of polyadenylated RNA hybridized with the wild type *cry 1C* gene probe;  
 (B): The same blot as in panel (A) reprobed with yeast NADPH-cytochrome P450 oxidoreductase specific probe.;  
 (C): RT-PCR of polyadenylated RNA with the primers 1CF and 1CR amplifying 492 bp. fragment at the 3' end of *cry 1C* gene (see Fig. 3-4)

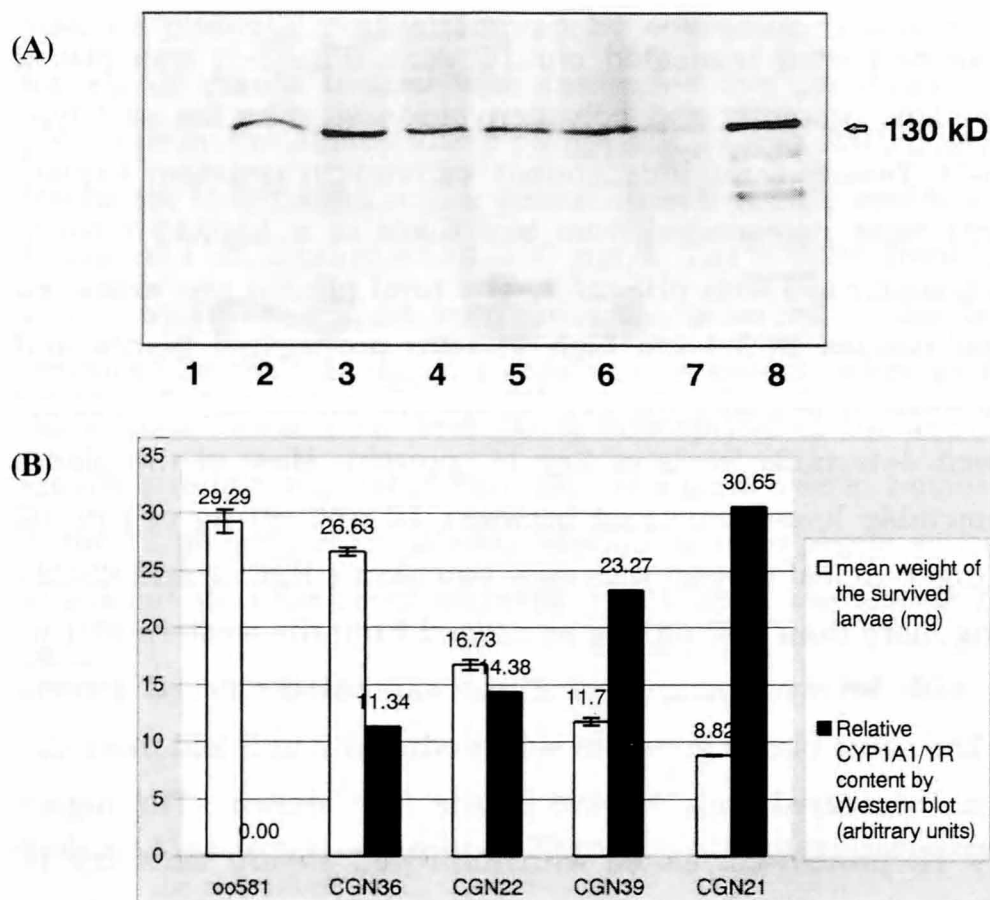


Fig. 3-6. The content of rat CYP1A1/yeast P450 oxidoreductase (CYP1A1/YR) protein and the insecticidal activity in the chlorotoluron resistant haploid tobacco plants coexpressing wild type *cry 1C* and *CYP1A1/YR* genes.

**(A):** Immunoblot detection of rat CYP1A1/YR fusion protein in the microsomes of pCGFN transformed tobacco plants. Fifty  $\mu$ g of crude microsomal protein fraction from transgenic tobacco plants were resolved in 6% SDS-PAGE and transferred to a PVDF membrane. The CYP1A1/YR fusion protein was detected with a Goat Anti-Rat CYP1A1 antiserum followed by Alkaline phosphatase labelled Rabbit Anti Goat Ab and visualized by staining in BCIP and NBT. Lane 1, untransformed control; lanes 2-6, transgenic plants CGN-19, CGN-21, CGN-22, CGN-36 and CGN-39; lane 7, Biorad<sup>®</sup> prestained protein standard (broad range); lane 8, microsomal proteins from yeast strain expressing CYP1A1/YR fusion protein.

**(B):** Graphical representation of the results from the bioassay experiments with neonate *S. litura* larvae and the CYP1A1/YR protein contents. Open bars represent the mean weight of the survived larvae after six days of feeding on the leaf tissues. and the shaded bars show CYP1A1/YR content in arbitrary units. The relative contents of CYP1A1/YR fused protein in tobacco microsome fractions were quantified from the western blot (A). Values for CYP1A1/YR protein were divided by 3 to fit the scale. Error bars represent the standard deviation between the three replications of the bioassay experiment with five larvae each (see the text for details).

### **3.3.4 Expression of the truncated synthetic *cry 1C* gene in tobacco plants**

The reconstructed truncated *cry 1C* gene (Fig. 3-1) was placed between the same promoter and polyadenylation sites as the wild type gene (Fig. 3-2). Twenty three independent kanamycin resistant haploid transformants were regenerated from leaf discs of a haploid tobacco Samson nn transformed with pBSynC1. The total protein was extracted from the leaf tissues of 3-4 cm high *in vitro* propagated plants and subjected to western blot with the Cry 1C specific antiserum. Ten of the plants showed detectable levels of Cry 1C protein. Most of the plants showing detectable levels produced between 10 and 50 ng of Cry 1C protein per mg total leaf protein with only two plants RSC22 and RSC23 accumulating more than 100 ng/mg as judged from the western blot by comparison with known amounts of *E coli* expressed Cry 1C protein (Fig. 3-7B). The same plants grown in soil produced 2 to 3 fold more Cry 1C protein as compared with *in vitro* plants (not shown). The higher levels of Cry 1C protein correlated with improved *steady state cry 1C* mRNA levels (compare western and northern blots in Fig. 3-7 and Fig. 3-8). The selected haploid tobacco plants were tested for insecticidal activity towards *S. litura* larvae in detached leaf bioassay. Ten neonate larvae of *S. litura* were infested on a leaf from soil grown tobacco plants in three replications and the mortality was recorded on the third and sixth days after infestation (Fig. 3-10). The selected plants with the exception of RSC3 caused between 80 and 100 % mortality of the larvae on the third day (Fig. 3-7B). The best expressing plant RSC23 accumulating more than 0.01 % of the leaf protein as Cry 1C killed all larvae allowed to feed on the leaf (Fig. 3-7 and Fig. 3-10). The tissue specific expression patterns of the Cry 1C protein was examined in the best expressing plant RSC23 by western blot. The Cry 1C protein was mainly accumulated in the leaf tissues, to a less extend in the stem and no protein cross-reacting with the anti Cry 1C antibody was detected in the root tissues (Fig. 3-9).

Production of double haploid tobacco plants transformed with the plasmid pBSynC1 was attempted by colchicine treatment. Out of 23 transgenic plants treated with colchicine five produced seeds. Seeds produced by the dihaploidized plants RSC21 and RSC23 were tested for resistance to kanamycin in a germination test on a medium, containing kanamycin at concentration 100 mg/l. The results from the test are shown in Table 3-3. As seen from the table all of the tested seeds, produced by the transgenic plants were resistant, whereas the seeds of the control Samson nn were 100% susceptible to the antibiotic. These results strongly suggested that the *npt ii* gene was in homozygous state in the T1 progeny of the double haploid tobacco plants which allows me to assume that the cotransformed *cry 1C* gene was also in homozygous state.

Table 3-3 Kanamycin resistance of the T1 progeny of tobacco plants, transformed with the plasmid pBSynC1

| plant ID  | <i>Total seeds tested</i> | <i>Resistant</i> | <i>Susceptible</i> | <i>Ratio R:S</i> |
|-----------|---------------------------|------------------|--------------------|------------------|
| Samson nn | 100                       | 0                | 100                | 0:1              |
| RSC21     | 100                       | 99               | 1                  | 1:0              |
| RSC23     | 100                       | 100              | 0                  | 1:0              |

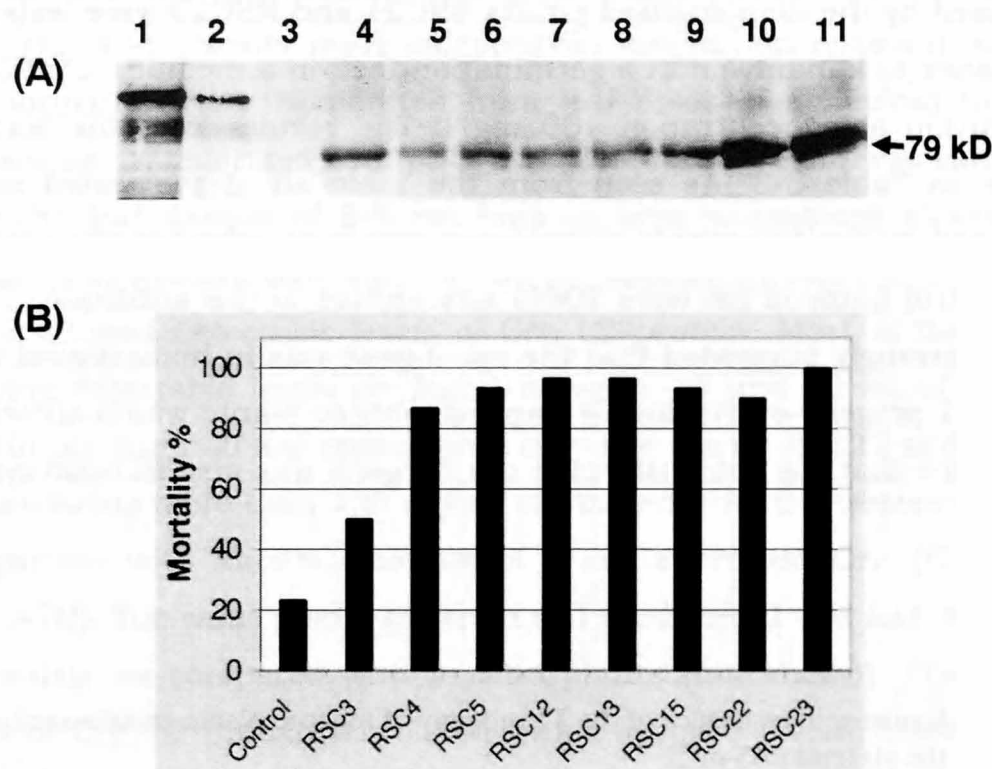


Fig. 3-7. Expression of the truncated synthetic *cry 1C* gene under the control of *rbcS* promoter in haploid tobacco plants.

(A): Western blot detection of the Cry 1C protein in tobacco plants. 20  $\mu$ g of total leaf protein were resolved in 8% SDS-PAGE and electrotransferred to PVDF membrane. The membrane was reacted with 1: 1000 diluted rabbit anti Cry 1C antiserum followed by 1:1000 dilution of AP conjugated Goat anti rabbit antibodies. Lane 1, 2 ng; lane 2, 0.2 ng truncated Cry 1C protein expressed in *E. coli*. The apparent  $M_r$  of the *E. coli* expressed truncated Cry 1C is higher due to additional 109 amino acids Trx-tag at the N-terminal of the protein. Lane 3 untransformed control; lanes 4-11 independent transformants with the plasmid pBSynC1: RSC3, RSC4, RSC5, RSC12, RSC13, RSC15, RSC22 and RSC 23, respectively.

(B): Mortality rate in bioassay experiment. Bioassay was done with 10 neonate *S. litura* larvae infested on a leaf from soil grown tobacco in three replications. The mortality was scored on the third day of incubation at 25 °C. Untransformed haploid tobacco Samson nn. was used as a control. RSC3 to RSC23, independent transformants with the plasmid pBSynC1.

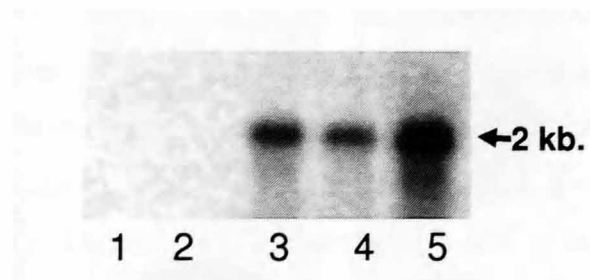


Fig. 3-8. Detection of the *cry 1C* transcripts in tobacco plants expressing the synthetic *cry 1C* gene by northern blot.

One  $\mu$ g polyadenylated RNA extracted from leaf tissues was separated in 1 % formaldehyde gel and transferred to Hybond N<sup>+</sup> membrane. Detection was done with [ $\alpha$ -<sup>32</sup>P] dCTP labeled fragment at the 3' end of the synthetic *cry 1C* gene. Lane 1, untransformed control; lane 2 tobacco plant transformed with the plasmid pRBS1CS (RC17), lanes 3-5, tobacco plants, transformed with pBSynC1, RSC3, RSC12 and RSC23 respectively.

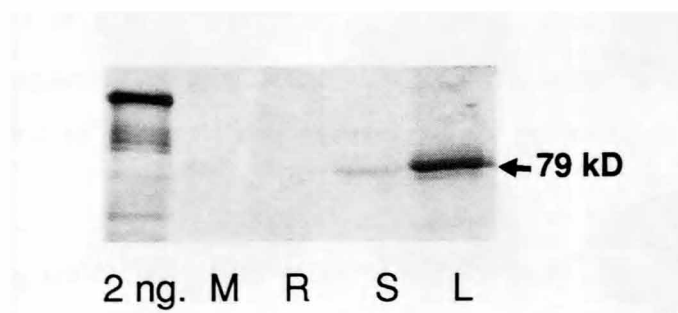


Fig. 3-9. Tissue specific expression of the Cry 1C protein in the transgenic tobacco line RSC23 expressing the synthetic *cry 1C* gene.

The total proteins from the root (R), stem (S) and leaf (L) tissues were extracted from the transgenic line RSC23, transformed with the plasmid pBSynC1. The extraction and the western blot were done as described for Fig 3-7. M is a molecular weight marker and 2 ng. is 2 ng. of *E. coli* expressed truncated Cry 1C protein.

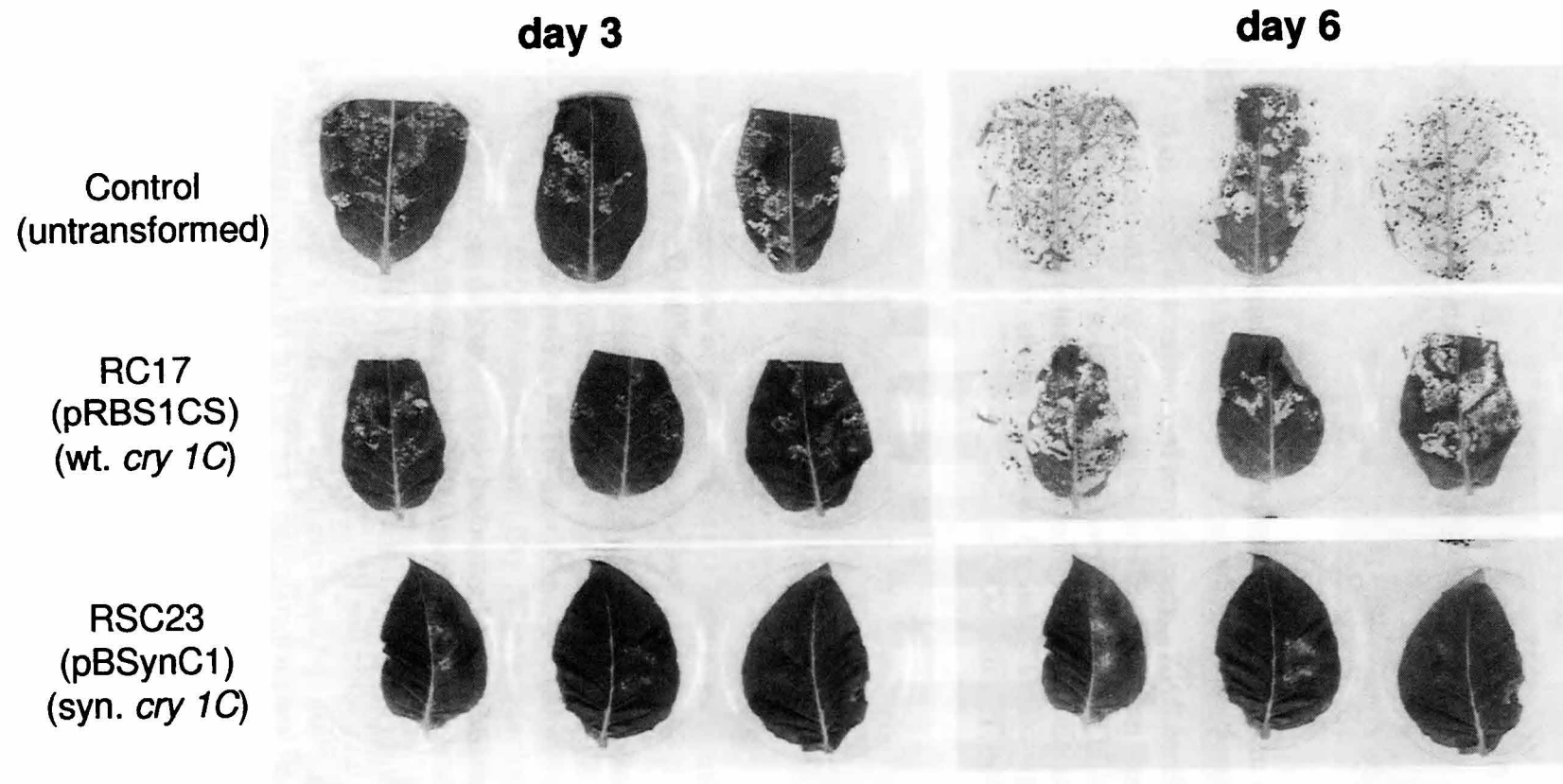


Fig. 3-10. Detached leaf bioassay of transgenic tobacco plants expressing each wild type *cry 1C* gene or synthetic *cry 1C* gene with neonate *Spodoptera litura* larvae.

Ten freshly hatched larvae of *S. litura* were infested per leaf from soil grown tobacco plants in three replications. The photographs were taken at the 3-rd and 6-th days of incubation at 25 °C, 70% RH. From top to bottom: untransformed control haploid tobacco *Samson nn.*; RC17, tobacco plant, transformed with the plasmid pRBS1CS; RSC23, tobacco plant, transformed with the plasmid pBSynC1.

### 3.4 DISCUSSION

The Cry 1C protein from *B. thuringiensis* is the most active to *Spodoptera* species among the Bt toxins tested. It is 20 to 40 fold more toxic to *Spodoptera* than the Cry 1Ab [49]. Moreover, the finding that the insect population of *Plutella xylostella* which developed resistance to Cry 1Ab and Cry 1Ac in the field retained the susceptibility to Cry 1C toxin [73], suggested the usefulness of the Cry 1C toxins in the insect resistance management strategies. The larvae of *Spodoptera litura* and other *Spodoptera* species susceptible to Cry 1C toxin feed primarily on the leaf of infested crop plants. So, the present study was focused on utilization of the green tissue specific expression of the *cry 1C* gene from *B. thuringiensis* in tobacco plants for the control of *S. litura* larvae. The previous attempts for the expression of native *cry* genes from *B. thuringiensis* showed strikingly low Cry protein levels and incomplete control of the target insects [18-21]. Complete reconstruction of the coding sequences of several *cry* genes to make them more plant-like led to a significant improvements of the Cry protein levels in the transgenic plants and control of most of the economically important pests [30, 33-35, 44, 45].

The coding sequence of the truncated *cry 1C* gene in the present study was also redesigned to conform to the highly expressed plant genes. The coding sequence was compared to the dicotyledonous RuBisCo small subunit (*rbcS*) cDNA sequences available in Gen Bank. The *rbcS* genes were chosen because they are highly expressed in the leaf tissues and have even more biased preference for some codons which might be required for high levels of tissue specific expression [54]. The comparison identified the codons in the *cry 1C* sequence which were rarely used in the plant *rbcS* genes and those were replaced in the reconstructed gene (Fig. 3-1). Sequence analyses of the reconstructed gene showed that most of the motifs, known to be

deleterious to the mRNA stability were automatically removed by the codon changes. The same phenomenon was noticed in other reconstructed *cry* genes and was attributed to the rise of the GC content and removal of the AT sequences motifs which may by chance contain motifs recognized by the plant as polyadenylation signals or intron border motifs as well as ATTTA sequences which target the mRNA for degradation [46].

The synthetic gene was constructed by a modified megaprimer PCR method in which 3 to 5 megaprimer oligonucleotides were simultaneously amplified in a single PCR reaction (Fig. 3-1). The simultaneous amplification of several oligonucleotides allowed to reduce the number of cycles required for the assembly of full length blocks as compared to the sequential PCR reactions and thus reduce the possibility of polymerase introduced errors. The specific design of the oligonucleotide megaprimers with short overlaps (Fig. 3-1) eliminated the need for purification of full length oligonucleotides and minimized the cost of the chemical synthesis. The recovery rate was somewhat variable for each block and appeared to depend on the quality of the crude oligonucleotide preparations. Analyses of the detected mutations revealed that most of them were at the overlaps between the individual oligonucleotides and were usually small deletions or insertions. Similar mutations were also found in the synthetic blocks, constructed by template directed ligation (TDL) PCR [44]. The Pfu ligase, used in the above mentioned study could not introduce mutations into DNA sequence and therefore it was concluded that the observed mutations were originated from the chemical oligonucleotide synthesis. The missincorporation of nucleotide residues is a common problem of the current chemical synthesis technology, especially when long oligonucleotides are being synthesized.

The tobacco plants transformed with the wild type *cry 1C* gene under the control of *rbcS* promoter showed insecticidal effects towards

neonate *S. litura* larvae expressed as inhibition of the larval development and weight reduction. The insecticidal effects of the selected plants correlated with the expression levels of the cotransformed *NPT II* gene, suggesting that the insecticidal effects were due to the expression of the *cry 1C* gene. The strong correlation between 1A1/YR protein contents and the insecticidal activity of the chlorotoluron selected plants transformed with the plasmid pCGFN provided additional evidence that the insecticidal activity was due to the expression of the *cry 1C* gene. Moreover, it implied on the usefulness of 1A1/YR as a selection marker for tobacco transformation which could be utilized not only for in vitro selection but also in the follow up breeding programs to facilitate the transfer and maintenance of the transgenes to other economically important cultivars. Although the *cry 1C* messenger was not detected in northern blots, the expression of the *cry 1C* mRNA in the leaf tissues of these plants was confirmed by RT-PCR. However, as it was reported by the other researchers, who transformed the wild type *cry 1C* genes in transgenic plants under the control of various promoters [39, 44, 63], the Cry 1C protein was below the detection limit of the western blot in the present study too. This result supports the view that the expression levels, sufficient to control *Spodoptera* species could not be achieved with the wild type *cry 1C* genes.

In contrast to the tobacco plants transformed with the wild type *cry 1C* gene, the plants, transformed with the reconstructed gene under the control of the same upstream and downstream sequences, produced higher Cry 1C protein levels which were easily detectable by western blot of the total leaf protein. As far as the Cry 1C protein was undetectable even in the best expressing plants transformed with the wild type gene, it was difficult to precisely estimate the exact levels of improvement. However, judging from the detection limit of the western blot, which was 10 ng. per mg. protein, the best plants transformed

with the reconstructed gene were estimated to produce over 10 fold improved levels of Cry 1C protein as compared with the wild type gene. The expression levels obtained in the present study, 0.01 % for *in vitro* plants and 0.02-0.03% of the total leaf protein in soil grown plants, were similar to that, reported for the synthetic *cry* 3A gene in potato under the control of *Mac* promoter [33] and the synthetic *cry* 1Ac gene in rice under the control of maize *ubiquitin* promoter [74]. Although sufficient to control *S. litura* larvae in bioassays (Fig. 3-7 and Fig. 3-10) the Cry 1C levels obtained in the present study were about ten fold lower than the levels reported for a synthetic *cry* 1C gene under the control of CaMV 35S promoter with four enhancer regions in tobacco and alfalfa [44]. The lower expression levels obtained in the present study may be due to the differences in the regulatory elements at the 5' and 3' of the gene coding sequence utilized in each study. Alternatively, the difference might be attributed to the "position effect" of the gene insertion. Over 200 fold differences in the mRNA levels for the same gene were reported depending on the specific site of insertion [66]. As far as only 23 transformants were obtained in the present study, it is possible that the number was not sufficient to select the rare events of integration in favor of high expression levels. The exact mechanisms which lead to the *cry* mRNAs instability in transgenic plants are not yet fully understood [46]. Comparison of partially modified *cry* 1Ab gene in which the motifs, known to interfere with the mRNA stability were removed and the fully reconstructed version of the same gene revealed 10 fold difference in the expression levels [30]. Therefore the possibility that some factors, deleterious to the expression of the *cry* 1C gene persisted in the reconstructed gene can not be completely ruled out.

Improved expression of the reconstructed *cry* 1C gene on mRNA level were also confirmed by northern blot. The northern blot (Fig. 3-8) showed clear signal of the expected size of 2 kb. and shorter fragments, reported in transgenic plants expressing a wild type *cry* 1Ac and *cry*

*1Ab* genes [28, 46] and partially modified *cry 3B* gene [75], were not detected. The higher mRNA levels correlated well with the higher Cry 1C protein levels in the independent transformants. Taken together these results suggest that the potential polyadenylation signals which could lead to premature polyadenylation and unstable shorter transcripts were removed from the gene coding sequence and the improvement of the *steady state* mRNA levels is responsible for the higher Cry 1C protein levels in the plants transformed with the reconstructed gene.

To determine the expression patterns of the Cry 1C protein in tobacco plants, leaf, stem and root tissues were examined by western blot. Although the Cry 1C protein seemed not to be efficiently imported into the chloroplasts (data not shown), it was specifically expressed in the leaf and stem tissues and was not found in the roots. Wong et al. [36] demonstrated by transient expression experiments with a synthetic *cry 1Ac* gene under the control of *rbcS* gene promoter from *Arabidopsis thaliana* that the import to the chloroplasts was not required for the high level expression of the transgene and the expression levels seemed to be dependent on the coding sequence.

Dihaploid seed progenies of two independent transgenic haploid tobacco plants expressing the synthetic *cry 1C* gene were produced by colchicine treatment. The T<sub>1</sub> seeds showed 100% resistance to Km in a germination assay. Therefore, the homozygous state of the *npt ii* gene was verified by the germination assay. The result suggested that the cotransformed *cry 1C* gene was also homozygous. In general it takes at least three generations of self fertilization to bring the genes transformed in a homozygous state. The dihaploidization by colchicine treatment of the haploid transgenic plants allowed production of plants homozygous for the transgene in just one generation. Such homozygous material is very important for rapid transfer of the transgenes to the economically important varieties in the follow up breeding programs.

To my knowledge the present study is the first attempt to utilize green tissue promoter for the expression of the wild type and the synthetic *cry 1C* genes in transgenic plants. The results, presented here demonstrated that the optimized green tissue specific expression of the *cry 1C* gene from *B. thuringiensis* in transgenic plants is a useful approach to accumulate the Cry 1C protein in the preferred feeding sites for the control of the rather resistant insect species such as *S. litura*.

## **Chapter IV**

### **Concluding remarks**



Insecticidal proteins of the soil bacteria *Bacillus thuringiensis* (Bt) are potent natural insecticides. A large number of transgenic crops expressing mainly Cry 1A type Bt toxins have been generated and some of them are readily commercialized for plant protection. Although very efficient for the control of some *Lepidoptera* species of economic importance, the Bt crops currently available on the market are still inefficient for control of the more resistant *Lepidoptera* species such as *Spodoptera litura*. There are two main reasons for the low efficiency of the commercial crops for control of the *S. litura*. The first is relatively high activity of the gut proteases of the larvae of this species to degrade the active fragment and the second is lower specificity of the Cry 1A toxins to the receptors on the midgut epithelial cells of *S. litura*.

Two approaches to generate transgenic tobacco plants expressing genetically engineered Bt genes with the improved efficiency for the control of *S. litura* were explored in the present study. The first approach addressed modification of protease sensitivity of the Cry 1Ab proteins. It was based on expression of truncated *cry 1Ab* gene with a mutated putative protease cleavage site.

The second approach was based on the tissue specific expression of the active part of a *cry 1C* gene which is the most active towards *Spodoptera* species among the Bt toxins known to date. In addition, the codon usage of the *cry 1C* gene was modified for improvement of the expression levels of the gene in dicot plant species.

In Chapter II engineering of *S. litura* resistant transgenic tobacco plants was attempted by the expression of truncated modified *cry 1Ab* gene in which three amino acids, Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>637</sub>, were replaced with Gln by site directed mutagenesis. The mutant protoxin was 2.5 times more active towards *S. litura* than the wild type protein when expressed in *E. coli* [51].

The truncated mutant and the wild type *cry 1Ab* genes coding for the first 647 amino acids were each placed under the control of *CaMV 35S* promoter and *nos* terminator in a binary vector, and were transformed into a haploid clone of *N. tabacum* cv. Samson nn via *Agrobacterium*. The presence of the transgene in the genomic DNA of tobacco plants was confirmed by PCR and Southern blot. Insecticidal activity of the transgenic plants was examined by infesting freshly hatched *S. litura* larvae on leaf disks from *in vitro* grown plants. The tobacco plants, transformed with the mutant and the wild type *cry 1Ab* gene showed each significantly improved resistance towards *S. litura* larvae compared to the untransformed control plants. On the other hand, no significant difference was found in the insecticidal effects between the plants transformed with the mutant and the wild type gene. One explanation for this result could be the extraordinary low expression levels which can mask the 2.5 fold difference in the toxicity of the mutant protein.

RT-PCR experiments revealed splicing of *cry 1Ab* genes at cryptic splicing sites which could not be predicted by sequence analyses. Based on these results it was concluded that the expression levels, high enough to effectively control the *S. litura* larvae could not be achieved by the expression of truncated wild type or the mutant *cry 1Ab* genes. The expression levels in tobacco plants were limited by the presence of cryptic splicing sites into the coding sequences of the genes which were difficult to predict even by the most sensitive currently available prediction methods.

In Chapter III engineering of *S. litura* resistance by tissue specific expression of the *cry 1C* gene from *B. thuringiensis* was attempted. A truncated *cry 1Ca5* gene coding for the active fragment (N-terminal 655 amino acids) of the protoxin was placed under the control of a tobacco *rbcS* promoter, which is one of the strongest tissue specific promoters and was transformed into tobacco plants. The transgenic tobacco plants transformed with the wild type gene

were characterized by PCR, RT-PCR and insect bioassays with the first instar of *S. litura* larvae.

The fusion gene between *rat CYP1A1* and *yeast P450 oxidoreductase (CYP1A1/YR)* which was shown to confer resistance to the herbicide chlorotoluron in transgenic tobacco plants [52] was also cotransformed with the *cry 1C* gene. The kanamycin resistant transgenic tobacco plants were subsequently selected for *in vitro* resistance to the herbicide chlorotoluron. The presence and the expression of both transgenes in the transformed tobacco plants were confirmed by PCR, northern blot, RT-PCR and western blot experiments, respectively. The selected tobacco plants were tested in bioassay experiments for the insecticidal effects towards the first instar *S. litura* larvae and for the resistance to the herbicide chlorotoluron.

The coding sequence of the truncated *cry 1C* gene was reconstructed for high level expression in tobacco plants by introduction of silent mutations which replaced 288 codons, rarely found in plant genes, and biased the codon usage of the *cry 1C* gene to resemble that of dicotyledonous *rbcS* genes. The reconstructed gene was assembled from crude synthetic oligonucleotides, ranging in length from 37 to 110 nt., by a modified megaprimer PCR method specially designed for the purpose. The synthetic *cry 1C* gene under the control of a green tissue specific promoter (*N. plumbaginifolia rbcS*) was transformed into tobacco plants. The transgenic tobacco plants, transformed with the synthetic gene were characterized by northern and western blot and were tested for insecticidal effect towards *S. litura* larvae in bioassay experiments. The tissues of tobacco plants transformed with the synthetic gene were examined for the Cry 1C protein expression patterns by western blot.

Seeds were produced after the colchicine treatment of two independent haploid tobacco plants, transformed with the synthetic *cry 1C* gene. The obtained T1 seeds were tested for kanamycin

resistance in seed germination assay. Based on these results the following conclusions were drawn:

There is no information in the literature on expression of wild type *cry 1C* genes from *B. thuringiensis* under the control of tissue specific promoters in transgenic plants. Therefore the present study is the first example for expression of the *cry 1C* genes in a tissue specific and light inducible manner into the green tissues of transgenic plants which are the main feeding target for *Spodoptera* larvae.

The Cry 1C protein is 10 to 20 fold more active towards *Spodoptera* than the Cry 1Ab protein. Therefore, it was concluded that the wild type *cry 1C* gene was expressed in the transgenic tobacco plants at levels even lower than the wild type *cry 1Ab*, regardless of the stronger promoter used. Direct comparison of the actual expression levels was prevented by the low, undetectable levels of mRNA and the corresponding protein of both wild type *cry 1C* and *cry 1Ab* genes in transgenic tobacco plants.

The present study for the first time provided a direct evidence that coexpression of the *CYP1A1/YR* was useful as an effective distal selection marker for *in vitro* selection of transgenic tobacco plants expressing the Cry 1C protein at high levels. Moreover such marker gene in contrast to the *npt ii* could be useful as a selection marker in the follow-up breeding programs in the field as resistance to the herbicide chlorotoluron.

A facile, time and cost effective megaprimer PCR method for construction of synthetic genes was invented and was successfully employed for the construction of the 1.9 kb. synthetic *cry 1C* gene.

The expression levels and the insecticidal activity of the transgenic tobacco plants, transformed with the synthetic *cry 1C* gene were significantly improved due to removal of the motifs which had deleterious effect on the mRNA processing and/or stability from the coding sequence of the synthetic gene. The Cry 1C protein was

specifically accumulated in the green tissues of tobacco plants transformed with the synthetic gene, suggesting that the strategy applied for the gene redesign for green tissue specific expression was successful.

The transgenes were stable inherited in homozygous state in the T<sub>1</sub> seed progeny of the dihaploidized transformed haploid tobacco plants. In general, it takes at least three generations in the follow-up breeding programs to bring the transgene transformed into crop plants in a homozygous state. The haploid transformation approach used in the present study could significantly speed-up the way from the laboratory to the field for the transgenic tobacco plants expressing novel types of Bt toxins.



## SUMMARY

*Spodoptera litura* is a polyphagous insect which is difficult to control due to the development of resistance to organophosphorus and pyrethroid insecticides. The control of this insect species with the transgenic plants expressing insecticidal proteins of *B. thuringiensis* is also limited by the relatively low susceptibility of the larvae to the Cry 1A type proteins, which were commonly expressed in transgenic plants. The present study aimed at the generation of *Spodoptera* resistant transgenic tobacco plants expressing genetically modified *cry 1Ab* and *cry 1C* genes from *B. thuringiensis* and of herbicide resistance plants expressing *rat CYP 1A1* fusion gene with *yeast NADPH cytochrome P450 oxidoreductase* as selection marker for tobacco transformation.

The truncated genes coding for the N-terminal 645 amino acids including the active fragment of the 2.5 fold more toxic to *S. litura* mutant version of Cry 1Ab protein, in which Arg<sub>619</sub>, Lys<sub>622</sub> and Lys<sub>629</sub> were replaced by Gln, and for the wild type protein were placed under the control of CaMV 35S promoter and *nos* terminator, and then transformed into tobacco plants. The integration of the *cry 1Ab* genes into tobacco genome was confirmed by Southern blot. Although some of the tobacco plants expressing each of the mutant and wild type genes were insecticidal to *S. litura* larvae, Cry 1Ab protein levels were low and undetectable by western blot. The RT-PCR experiments revealed that the *cry 1Ab* transcripts were spliced at cryptic intron splicing sites in the transgenic tobacco plants.

The truncated version of a *cry 1C* gene under the control of a tobacco *rbcS* promoter, which is one of the strongest tissue specific promoters, was transformed into tobacco plants.

The fusion gene between rat CYP1A1 and yeast P450 oxidoreductase (CYP1A1/YR) which was shown to confer resistance to the herbicide chlorotoluron in transgenic tobacco plants [52] was cotransformed with the *cry 1C* gene. The kanamycin resistant transgenic tobacco plants were subsequently selected for *in vitro* resistance to the herbicide chlorotoluron. The selected tobacco plants inhibited the development of the insect larvae. The insecticidal effects of chlorotoluron resistant tobacco plants towards *S. litura* correlated well with CYP1A1/YR protein accumulation. The herbicide resistance of the transgenic tobacco plants indicated that the CYP1A1/YR is a useful selection marker for tobacco transformation as resistance to the herbicide chlorotoluron.

The coding sequence of the truncated *cry 1C* gene was reconstructed for high level expression in tobacco plants by introduction of silent mutations which replaced 288 codons, rarely found in plant genes, and biased the codon usage of the *cry 1C* gene to resemble that of dicotyledonous *rbcS* genes. Most of the known mRNA instability determinants present in the wt. *cry 1C* gene were automatically eliminated by the codon replacement. The reconstructed gene was assembled from crude synthetic oligonucleotides ranging in length from 37 to 110 nt. by a megaprimer PCR method. The synthetic *cry 1C* gene under the control of a green tissue specific promoter (*N. plumbaginifolia rbcS*) was transformed into tobacco plants. In contrast to the plants transformed with the wild type gene which showed undetectable levels of *cry 1C* mRNA and the corresponding protein as well as marginal protection toward *S. litura* larvae, the plants expressing the synthesized gene showed significantly improved of *cry 1C* protein and mRNA levels. The tobacco plants transformed with the synthesized gene which produced more than 0.01% of the total leaf protein as Cry 1C showed complete control of the *S. litura* larvae in detached leaf bioassays. Examination of the tissues of tobacco plants transformed with the synthetic gene by western blot revealed that the

Cry 1C protein was primary accumulated in the green tissues (Leaf and Stem) and was undetectable in the roots.

Seeds were produced after colchicine treatment of two independent haploid transformants expressing the synthetic *cry 1C* gene. The results of the germination tests of the seed progeny suggested homozygous state of the cotransformed *npt ii* marker gene and the linked *cry 1C gene* in the seed progeny.

The results, reported here demonstrated that the optimized green tissue specific expression of the synthetic *cry 1C* gene in transgenic plants is useful for control of *S. litura* species which are resistant to the other types of Bt toxins.

## ACKNOWLEDGMENTS

I am most grateful to Professor Hideo Ohkawa of Kobe University for his continuous guidance, stimulating discussion and encouragement throughout the course of my graduate program, and for the critical reading of my dissertation.

I am very grateful to Associated Professor Hiromasa Imaishi and Dr. Noriaki Shiota of Kobe University for teaching me the experimental methods in molecular biology, and for their valuable advice and discussion during my experimental work.

I wish to express my gratitude to Associated Professor Masanobu Nakata of Kobe University, for his advice and support throughout this work.

I am deeply indebted to Mr. Hideyuki Inui for the useful discussions and help with the document translations.

I thank to Associate Professor Yasuo Fukami, Assistant Professor Ken-ichi Sato and Assistant Professor Alexander Tokmakov for allowing me to use the facilities of the Laboratory of Molecular Biology of Kobe University.

I acknowledge Dr Nam Hai Chua of Rockefeller University for providing the *rbcS* promoter of *N. plumbaginifolia*, Dr. Takashi Yamamoto of Sandoz Agro, Inc., for the plasmid containing the *cry 1C* gene from *B. thuringiensis*, and Dr. Yasunori Shono of Sumitomo Chemical Co., for providing the eggs mass of *S. litura*.

My special thanks to my colleagues in Laboratory of Agrochemical Biochemistry, Kobe University, for their help, kindness and understanding.

I am grateful to the Ministry of Education, Science, Sports and Culture of Japan (MONBUSHO), for supporting my research and stay in Japan.

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# PUBLICATIONS

## Chapter II

Nikolai K. Christov, Noriaki Shiota, Hideo Ohkawa

*Spodoptera litura* tolerant transgenic tobacco plants expressing genetically modified *cry 1 Ab* genes from *Bacillus thuringiensis*, (1998) *Biotechnology & Biotechnol. Eq.*, **1**, pp.8-16

## Chapter III

Nikolai K. Christov, Hiromasa Imaishi, Hideo Ohkawa

Green tissue specific expression of a reconstructed *cry 1C* gene coding for the active fragment of *Bacillus thuringiensis*  $\delta$ -endotoxin confers resistance to *Spodoptera litura* in haploid tobacco plants, (1999) *Biosci. Biotech. Biochem.*, *submitted*