

**RESPONSE OF TRANSGENIC RICE PLANTS
TO RICE LEAFFOLDER, *Cnaphalocrocis
medinalis* (Guenee) AND STEM BORER,
Scirpophaga incertulas (Walker)**

By

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the degree of :

DOCTOR OF PHILOSOPHY

IN

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**COLLEGE OF AGRICULTURE,
CCS HARYANA AGRICULTURAL UNIVERSITY,
HISAR**

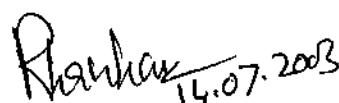
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- TO MY LOVING HUSBAND

CERTIFICATE – I

This is to certify that this thesis entitled, “Response of transgenic rice plants to rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and stem borer, *Scirpophaga incertulas* (Walker)”, submitted for the degree of Ph.D., in the subject of Entomology of CCS Haryana Agricultural University, Hisar, is a bonafide research work carried out by Ms. Rekha Sagwal under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.



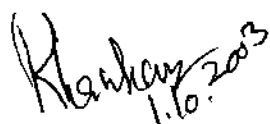
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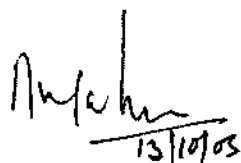
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CHAPTER – I

INTRODUCTION

Rice, *Oryza sativa* (L.) is one of the most important cereal crops in the world and the loss of rice yield due to direct insect damage is estimated to cost several billion dollars per year. At least 15 per cent of world's crop yields are lost due to insect damage, with further losses attributed to plant diseases for which insects serve as the transmission vectors (Heinrichs *et al.*, 1985). The rice plant is subjected to attack by more than 100 species of insects; 20 of them cause economic damage (Pathak and Khan, 1994). Thus, among the various factors responsible for its lower productivity, insect pests are one of the important constraints.

The rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and yellow stem borer, *Scirpophaga incertulas* (Walker) which are the most important pests of rice, have now become widespread throughout the major rice growing regions of Asia (Khan *et al.*, 1989). Synthetic chemical insecticides have been used for the protection of rice plants from pest damage. Such insecticides have caused environmental pollution, insect resurgence, as well as insecticide resistance. Therefore, the chemical control of pests is under increasing pressure. Pesticide

use in the world and specifically in European countries is currently declining, largely due to regulatory mechanisms, environmental activism and public pressure. This has necessitated the use of target specific compounds with low persistence, and an increase in emphasis on integrated pest management (IPM) based on host plant resistance to insect pests. Although the benefits to agriculture from pesticide use to prevent insect associated losses cannot be overlooked, but there is a greater need to develop alternative or additional technologies, which would allow a rational use of pesticides, and provide adequate crop protection for sustainable food, feed and fibre production in the future.

Host plant resistance is an integral part of integrated pest management. However, conventional host plant resistance to insects involves quantitative traits at several loci, and as a result, the progress has been slow and difficult to achieve. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert genes into the plant genome that confer resistance to insects (Bennett, 1994). Therefore, the increasing demand to use non-hazardous, environmentally compatible pest control measures has necessitated the use of ecofriendly insecticides such as *Bacillus thuringiensis* (Bt) insecticidal crystal proteins and plant-derived insecticidal proteinase inhibitors (Vaeck *et al.*, 1987; Boulter *et al.*, 1989; Ryan, 1990; Gill *et al.*, 1992; Charles *et al.*, 1996). A common feature of many of these compounds is that they have a chronic rather

than an acute toxicity on insects and their effects are less dramatic than those of the synthetic insecticides.

Transformation approaches offer an attractive alternative to the conventional techniques to introduce one or more useful genes to improve rice without disrupting desirable genetic make-up. Insect-resistant transgenes, whether of plant, bacterial or other origin, can be introduced into plants to increase the level of insect resistance, a technology that has dramatically extended the scope of resistance genes available to plant breeders. The first report of transgenic insect-resistant plants was only published in 1987 (Hilder *et al.*, 1987; Vaeck *et al.*, 1987). Approximately 40 different genes conferring insect resistance have been incorporated into crops (Schuler *et al.*, 1998). This new technology is seen as an additional tool for the control of crop pests and could offer certain advantages over conventional insecticides, such as more-effective targetting of insects within plants, greater resilience to weather conditions, fast biodegradability, reduced operator exposure to toxins and financial savings (Gatehouse *et al.*, 1992). The use of transgenic plants may lead to a reduction in the use of broad spectrum insecticides, thereby extending the useful life of these compounds and reducing the ecological damage they cause (Schuler *et al.*, 1998). However, there is concern that constitutive expression of toxins will encourage the selection of resistance to such products in pest populations (Roush, 1997).

Transgenic plants rarely result in 100 per cent control, but tend to retard insect development and growth (Estruch *et al.*, 1997). Plant-derived genes attack different sites in insects than the synthetic chemicals, and may be deployed in combination with exotic genes and insecticides. Retardation of insect development, slower rate of insect population growth and reduced fitness of the surviving insects would allow a much wider window within which intervention with insecticides can be successfully employed. This will help to generate confidence in the IPM by the farmers, who normally prefer complete insect control based on chemical pesticides.

Plant-derived proteinase inhibitors are another class of protein, which confer insect resistance to plants by disturbing insect luminal food digestion. Previous studies (Ryan, 1989; Ryan, 1990; Duan *et al.*, 1996; Xu *et al.*, 1996) on the effects of dietary proteinase inhibitors have shown that these proteinase inhibitors can be detrimental to the growth and development of a wide range of insects. Since, these proteinase inhibitors are common components of plant-derived food for human and animals and that they are easily inactivated by cooking, introduction of proteinase inhibitor gene into new host crops has been regarded as a safe strategy for insect control from the food safety point of view.

Transgenic plants with insecticidal genes are set to feature prominently in pest management in both developed and the developing countries in the future (Roush, 1997). Entomologists, breeders and the molecular biologists need to

determine how to deploy this technology for pest management, and at the same time reducing possible environmental hazards. To achieve these objectives, we need to have a proper understanding of the insect biology, behaviour, their response to the insecticidal proteins, temporal and spatial expression of insecticidal proteins in the plants, strategy for resistance management, impact of insecticidal proteins on natural enemies and non-target organisms and a mechanism to deliver the technology to the resource-poor farmers (Sharma *et al.*, 2000).

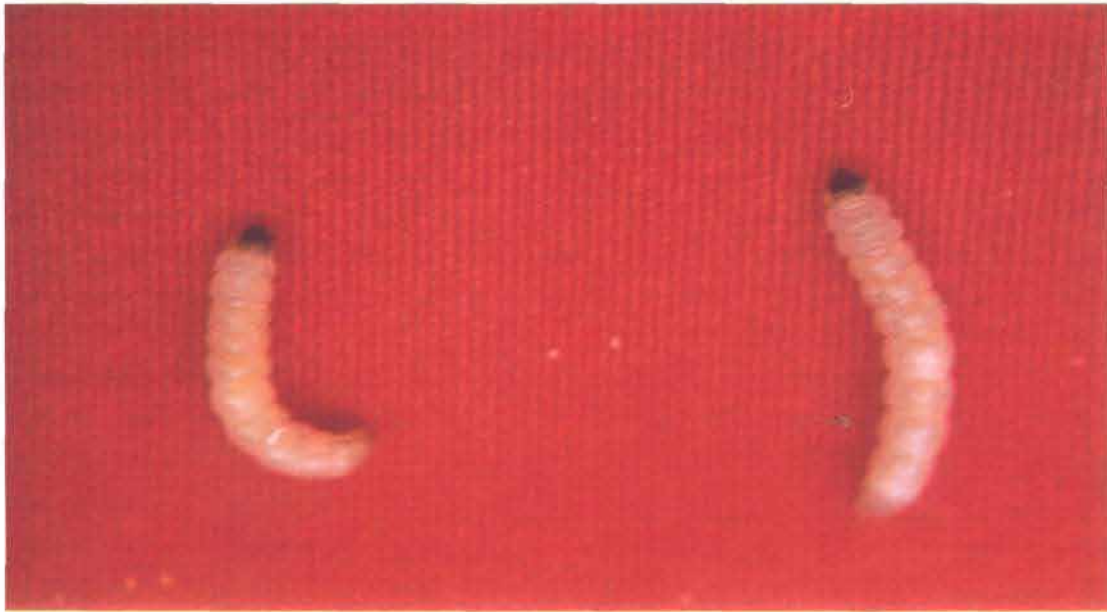
Keeping the above facts in view, the present studies were proposed to be carried out on transgenic rice (which possess proteinase inhibitor gene) and its mechanisms of resistance against rice leaffolder and stem borer.

Objectives:

1. Evaluation of transgenic rice plants for *pin II* gene (Potato Proteinase inhibitor-II) by PCR (Polymerase Chain Reaction) analysis.
2. Evaluation of transgenic rice plants for response against rice leaffolder and stem borer by leaf-section and whole plant bioassays.
3. To study the biology of rice leaffolder, *Cnaphalocrocis medinalis* on transgenic rice plants.
4. To establish the mechanisms of resistance in transgenic rice plant against rice leaffolder



Cnaphalocrocis medinalis (Guenee) larva



Scirpophaga incertulas (Walker) larvae



Leaffolder damage



Stem borer damage at earhead stage of rice plant

CHAPTER – II

REVIEW OF LITERATURE

Insects not only cause direct loss to the agricultural produce, but also indirectly as vectors of various plant pathogens. In addition to direct losses caused by insects, there are additional costs in the form of pesticides applied for pest control. Rice is one of the most important cereal crops in the World being grown in 145 million ha with a production of over 468 million tons (Bhutani, 2001).

2.1 Important Insect Pests

A variety of insect pests cause severe damage to rice production in many Asian countries. In Asia, lepidopterous stem borers, such as yellow stem borer, *Scirpophaga incertulas* (Walker) causes economic damage to rice, *Oryza sativa* L. Stem borers are important pests of rice because symptoms of injury can be found from sowing to harvest in all rice ecosystems. At the vegetative stage, stem borer feeding inside the stem, can result in the death of the youngest leaf whorl, causing “dead hearts”. During the reproductive stage, stem borers feeding inside the panicle stalk can lead to grain remaining unfilled, a condition called “white heads” (Rubia *et al.*, 1995). Average annual yield losses are often estimated at 5-10 per cent and occasionally as much as 60-95 per cent (Pathak and Khan, 1994).

Rice foliage is also attacked by other lepidopterous species in Asia, including rice leaffolder, *Cnaphalocrocis medinalis* (Guenee). Rice leaffolder is an important pest in almost all of the rice growing countries of Asia (Khan *et al.*, 1988). Among the four leaffolder species recorded in India, *C. medinalis* is widely spread and causes significant yield losses (Pandya *et al.*, 1987; Shrivastava, 1989). The larvae fold the leaves and scrap the green mesophyll tissue of the leaf, resulting in reduction of the photosynthetic activity of leaves. The scraped leaves become membranous, turn white and finally wither. The heavily infested crop has streaks on the leaves and appears whitish from a distance.

2.2 The Agrochemical 'Solution'

The conventional control of insect pests in rice cultivation often depends on the use of chemically synthesized insecticides which is associated with adverse effects on the beneficial organisms, environmental pollution, insect resurgence as well as insecticide resistance. As a result, the chemical control of pests is under increasing pressure. To reduce the use of chemical insecticides, one option is to use resistant rice varieties. However, despite extensive screening, high levels of natural resistance to stem borers and rice leaffolder have not been found in rice germplasms (Heinrichs *et al.*, 1985; Khan *et al.*, 1991; Jackson, 1995). Thus, the need to use non hazardous, environmentally compatible pest control measures has spurred an interest in natural insecticides such as *Bacillus thuringiensis* (Bt) insecticidal crystal proteins (Fischhoff *et al.*, 1987), protease inhibitors, plant

lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from Bt and related species and small RNA viruses and these can be used alone or also in combination with Bt genes to generate transgenic plants for pest control (Hilder and Boulter, 1999).

2.2.1 Transgenic Approaches

Transgenic approaches offer powerful means for providing insect resistance by incorporating insect resistance genes (Duan *et al.*, 1996; Sharma *et al.*, 2000). There are several advantages of using transgenic plants for insect control as opposed to breeding for insect resistance from related varieties or ancestors of crop plants. Transgenic approach is useful in the absence of effective host plant resistance to many insects like stem borer resistance in rice. It allows the addition of specific character into rice without the linkage drag and the requirement for backcrossing that accompany sexual hybridization. The direct introduction of a single dominant gene into commercial rice varieties has additional advantages over breeding for a multigene trait (Duck and Evolva, 1996).

2.2.1.1 Use of Plant-derived genes

Plant-derived proteinase inhibitors are of particular interest because these are part of the natural plant defense system against insect infestation (Mochizuki *et al.*, 1999). When foreign plant proteinase inhibitor genes were introduced into tobacco, transgenic tobacco plants expressing the proteinase inhibitor proteins at relatively high levels were found resistant to typical tobacco insect pests (Hilder *et*

al., 1987; Johnson *et al.*, 1989). Expression of protease inhibitor genes has been useful for the control of insects, which feed by chewing plant tissues such as insects belonging to Lepidoptera and Coleoptera. It is advantageous to use proteinase inhibitors as insect control agents because they are active against a wide range of insects, and they also act as insect control agents for insects that are resistant to the Bt endotoxin. However, high levels of protein inhibitors are required for insect killing and there is potential need to regulate expression of such inhibitors to specific plant organs. Duan *et al.* (1996) and Xu *et al.* (1996) produced transgenic Japonica rice plants containing potato proteinase inhibitor II (*pin II*) and cowpea trypsin inhibitor (CpTI) genes respectively. Transgenic plants had high level of accumulation of the *pin II*/CpTI proteins and their progenies showed increased resistance against pink stem borer.

2.3 Proteinase Inhibitor (Introduction)

Proteins that form complexes with proteases and inhibit their proteolytic activity are widespread in nature. The synthesis of antimetabolic proteins, which interfere with the digestive processes in insects, is a defense strategy that plants use extensively. Such proteins can be synthesized constitutively in tissues that are particularly vulnerable to attack, such as, seeds or can be induced by mechanical wounding, as it is the case when chewing-insects feed on leaves. The defensive role of enzyme inhibitors in protecting plants against insect pests also exists in many cases (Ryan, 1990).

The proteinase inhibitors in plants have been known since 1938 and they are extremely widespread in their distribution throughout the plant kingdom. They have been studied most extensively in the Leguminosae, Gramineae and Solanaceae, probably because of the large number of species in these families which form important sources of food (Richardson, 1977).

Plant proteinase inhibitors (PIs) are polypeptides or proteins that occur naturally in a wide range of plants and are a part of the plant's natural defense system against herbivore. Proteinases in insects include serine, cysteine, aspartic and metallo proteinases that catalyse the release of amino acids from dietary protein and so provide the nutrients crucial for normal growth and development (Wolfson and Murdock, 1990a). In plants particularly eight and possibly ten protease-inhibitors families have been recognized *viz.*, soybean trypsin inhibitor (Kunitz family), Bowman-Birk inhibitor family, barley trypsin inhibitor family, potato inhibitor I family, potato inhibitor II family, squash inhibitor family, ragi 1-2/maize bifunctional inhibitor family, carboxypeptidase A, B inhibitor family, cysteine proteinase inhibitor family (Cystatins), aspartyl proteinase inhibitor family (Ryan, 1990; Reeck *et al.*, 1997).

2.4 Mechanism of Antimetabolic Action of Digestive Enzyme Inhibitors

Protein digestion is primarily accomplished extracellularly in animals, such as in the stomach, intestine, rumen or midguts of insects. When protease inhibitors are present in high concentration in foods of some farm animals (Liener

et al., 1988), they can significantly alter digestive processes and interfere with the growth and development. Protease inhibitors do not pose a direct problem for humans, because foods that contain high levels of these proteins are cooked, which inactivates the inhibitors. Ryan (1990) suggested that the presence of high levels of proteinase inhibitors on a continual basis can lead to chronic hypersecretion by the pancreas, loss of proteolytic activity in the gut, loss of appetite, starvation and eventual death.

Interfering with digestion, and thus affecting the nutritional status of the insect, is a strategy widely employed by plants to defend themselves against pests. A major factor in inhibition of digestion is the presence of protein inhibitors of digestive enzymes (both proteinases and amylases) in the plant tissues. These proteins interact with digestive enzymes, binding tightly to the active site and preventing access of the normal substrates (Garcia-Olmedo *et al.*, 1987). In case of proteinase inhibitors, binding is accompanied by hydrolysis of a target peptide bond in the inhibitor, which determines its specificity toward a particular type of protease. The enzyme inhibitor complex is both thermodynamically and kinetically very stable and thus stoichiometric inhibition of the enzyme is achieved. The inhibition of digestive enzymes not only has a direct effect on insect nutritional status, but is also thought to lead to secondary effects where oversynthesis of digestive enzymes occurs as a feedback mechanism in an attempt to utilize ingested food, if the insect cannot overcome the inhibition of digestion,

death by starvation occurs (Gatehouse and Gatehouse, 2000). Wolfson and Murdock (1987) and Jongsma and Bolter (1997) also reported the same mechanism of antimetabolic action of proteinase inhibitors. There are reports in case of mammals that when proteinase inhibitors enter the intestinal lumen, they irreversibly bind to the proteases which results in either increase production of proteases by pancreas or prevent reabsorption of inhibited enzymes (Broadway and Duffey, 1986).

Boulter (1993) reported that the gut digestive enzymes are not only the target affected by proteinase inhibitors, they can also affect the water balance, moulting and enzyme regulation.

2.5 Proteinase Inhibitors (PIs) Synthesis in Plant Tissues

The naturally occurring proteinase inhibitors in plant tissues are usually found in high concentrations in plant storage organs such as seeds or tubers including the staple crops, cereals, beans and potatoes (5-15% of total protein) (Jongsma and Bolter, 1997). The tubers of potato (*Solanum tuberosum* L.) are a notable source of a wide diversity of proteinase inhibitors where these accumulate during tuber development alongwith the potato inhibitor I and II families of serine proteinase inhibitors. In this tissue, there are atleast seven different types of proteinase inhibitors which can be distinguished on the basis of their varying molecular weights, amino acid compositions, N- and C-terminal amino acids, stability during heating and specificity. The inhibitors also accumulate in potato

leaf tissues, alongwith inhibitor I and II proteins, in response to wounding. Thus, wounded leaf tissues and potato tuber tissues have the capacity to inhibit all five of major digestive enzymes of higher animals and many insects (i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B) (Richardson, 1977; Ryan, 1990).

The proteinase inhibitors (PIs) have long been known as anti-nutritional factors that negatively affect the growth of young animals. Leaves normally contain very low PIs levels, but Green and Ryan (1972) found the PIs were actively induced to high levels in leaves of potato and tomato plants when they were attacked by Colorado potato beetle or mechanically damaged. To a local synthesis of PIs it was found that signals from the wound site were transported through the phloem and stimulated the synthesis of PIs throughout the plant.

Wolfson and Murdock (1990b) found that a systemic accumulation of PIs in non-wounded plant parts diminished in aging plants and did not occur at all in mature plants over 30 day old. However, daily wounding and insect feeding on mature tobacco and tomato plants did result in a rapid increase in trypsin inhibitor levels in the wounded leaf itself indicating that (Jongsma *et al.*, 1994; Alarcon and Malone, 1995) PIs can be induced locally by wounding throughout the plant's lifespan but the ability to induce PIs systemically in all the leaves is apparently lost as the plant matures. Jongsma *et al.* (1994) also reported that even though wounding a lower leaf of a mature plant did not lead to PI synthesis in upper

unwounded leaves, wounding lower and upper leaves at once did lead to a much more rapid PI accumulation in the upper leaf.

Duan *et al.* (1996) recorded that the level of proteinase inhibitors increased in successive generation. They introduced the potato proteinase inhibitor II (*pin II*) gene into several Japonica rice varieties and regenerated a large number of transgenic rice plants. The *pin II* protein levels after wounding the R₁ plants (1st generation plants containing *pin II* gene) were analyzed. Among those R₁ plants, 65 per cent produced similar *pin II* protein level to their R₀ parents (0-generation) and 35 per cent produced twice as much *pin II* protein as their R₀ parents. The R₂ plants accumulated equally high levels of *pin II* proteins as their R₁ parents and they all contain approximately twice the level of *pin II* as their R₀ parent.

Some workers (Ryan, 1968; Green and Ryan, 1972; Bryant *et al.*, 1976) suggested that wound-induced accumulation of proteinase inhibitors in potato and tomato leaves is mediated by a wound hormone, called proteinase-inhibitor-inducing factor (PIIF), that is released from severely wounded tissues and travels through the vascular system to both nearby and distal tissues where it initiates the accumulation of proteinase inhibitors I and II. Walker-Simmons and Ryan (1977) reported that PIIF wound hormone accumulated the highest level of trypsin inhibitory activity in alfalfa leaves. Three solonaceous plants, tobacco, tomato and potato followed in that order in trypsin inhibitor activity due to PIIF while

strawberry, cucumber, squash, grape, clover and broadbean accumulated low but significant trypsin inhibitory activity.

According to Cortes *et al.* (1989), potato and tomato plants accumulate proteinase inhibitor I and II in leaves as a direct consequence of insect damage or mechanical wounding. Both inhibitors are specifically directed against insect proteases and are considered to be part of the natural defense mechanism of plants against insects (Cortes *et al.*, 1988; Green and Ryan, 1972). The accumulation of these proteins is not restricted to the wound site, but it is also observed in non-wounded aerial tissues. This indicates that upon wounding, wound inducing factor or wound hormone is released and by the way of vascular system, it is rapidly transported to other tissues of the plant, inducing the expression of these proteinase inhibitor genes. Graham *et al.* (1985) observed from their studies that pectic polysacchrides derived from the cell wall have been shown to be powerful inducers of the proteinase inhibitor gene when supplied to the excised leaves through cut petiole and have thus been postulated as a possible proteinase inhibitor inducing factor. However, Cortes *et al.* (1989) reported that the hormone Abscisic acid (ABA) is directly involved in the wound induction of the proteinase inhibitor-II genes. When ABA directly sprayed on to the leaves of potato, it proved to be sufficient to induce the accumulation of PI-II mRNA as a result of wounding.

2.6 Insect Bioassay on Proteinase Inhibitors

A major goal of plant genetic engineering is the introduction of agronomically desirable phenotypic traits into crop plants in situations where conventional breeding methods have been unsuccessful. One such target is enhanced resistance to insect pests which in view of the estimated production losses world-wide and the heavy costs of protective treatments is very important (Hilder *et al.*, 1987).

The effects of dietary proteinase inhibitors, either artificially introduced into defined diets or already present in plant tissues, have shown that these proteinase inhibitors can be detrimental to the growth and development of wide range of insects (Ryan, 1989, 1990). Proteinase inhibitors have been implicated as possible plant defense compounds (Lipke *et al.*, 1954; Applebaum, 1964) before they were known to be inducible. The potential of an induced proteinase inhibitor to affect plant's herbivores depends upon many factors; among them the susceptibility of the herbivores, digestive proteinases to inhibition by the plant's proteinase inhibitor and the plant's capacity to produce the inhibitors under the environmental conditions that accompany herbivore (Wolfson and Murdock, 1990b).

The role of plant proteinase inhibitors as a defense against insects is based on the increased levels of protease inhibitors detected in plants that have been damaged by insect feeding (Green and Ryan, 1972) and the extensive distribution

of inhibitors in plants (Liener and Kakade, 1980). Although two orders of phytophagous insects, Lepidoptera and Coleoptera, potentially ingest these antimetabolites, their effect on larval growth and development depends on the insect and inhibitor used and the results are variable (Jongsma and Bolter, 1997). In most plants, natural mechanisms to control insect attack include protease inhibitor accumulation (Ryan, 1973). Addition of different protease inhibitors to insect diets and their mechanism of action and effect on the growth and digestive physiology have been shown to limit insect development (Gatehouse *et al.*, 1980; Shukle and Murdock, 1983; Larocque and Houseman, 1990). Thus the over expression of PIs in transgenic plants may decrease insect damage (Hilder *et al.*, 1987; Johnson *et al.*, 1989).

The first example of the use of a plant-derived insect control protein gene in transgenic plants comes with the constitutive expression (from the CaMV 35S gene promoter) of a trypsin inhibitor gene derived from cowpea (*Vigna unguiculata*) in tobacco against tobacco budworm (*Heliothis virescens*), the results depicted as increased mortality, reduced growth and reduced plant damage (Hilder *et al.*, 1987). Gatehouse and Hilder (1994) proposed that the most active inhibitor identified to date is the cowpea trypsin inhibitor (CpTI), which has been transferred into at least ten other plant species. CpTI affects a wide range of lepidopteran and coleopteran species and conferred significantly enhanced level of resistance (Xu *et al.*, 1996). Hoffmann *et al.* (1992) reported that in California,

CpTI transformed tobacco caused significant mortality of cotton bollworm (*Helicoverpa zea*) larvae, but the protection provided by CpTI was less pronounced and consistent than that of tobacco containing a truncated Bt-toxin gene. Serine and cysteine proteinase inhibitors have also been reported to inhibit the growth and development of a range of insects, mainly lepidopteran and coleopteran species, respectively (Broadway and Duffey, 1986; Orr *et al.*, 1994). This and various other protease inhibitor gene constructs have been introduced in to a variety of different transgenic plants (Schuler *et al.*, 1998; Hilder and Boulter, 1999).

Earlier workers have studied the effect of plant proteinase inhibitors when ingested by the insects through artificial diets. Broadway and Duffey (1986) have shown that the plant PIs- soybean trypsin inhibitor (STI) and potato proteinase inhibitors II can significantly reduce growth and development of *H. zea* and *Spodoptera exigua* larvae. This retardation of growth is not directly due to a reduction in the levels of digested protein, rather the chronic ingestion of PIs induces the secretion of significantly higher levels of trypsin. This pernicious hyperproduction of the primary larval proteolytic enzyme proves detrimental to the larval noctuids.

Wolfson and Murdock (1987) suggested that growth and development of Colorado potato beetle (CPB) larvae was negatively affected by chronic ingestion of a cysteine proteinase inhibitor (E-64) when the larvae ingested E-64 at a rate as

low as 0.8 $\mu\text{g}/\text{cm}^2$. It could bind irreversibly with the cysteine proteinase and thereby decrease protein digestion directly. Alternatively, it could bind with the cysteine proteinase and via feedback mechanism initiate increased production of proteinase, resulting in wasted energy and amino acid utilization. Cysteine proteinase inhibitor (E-64) prolonged also the developmental time and increased the larval mortality of cowpea weevil, *Callosobruchus maculatus* (F.) when incorporated into the artificial seeds at low levels (0.01-0.25% by wt.) (Murdock *et al.*, 1988; Hines *et al.*, 1990; Bolter and Green, 1997).

Wolfson and Murdock (1995) studied the effect of cysteine protease inhibitor (E-64) on the life parameters of Mexican bean beetle, *Epilacna varivestis* Mulsant. Consumption of low doses of E-64 had a negative effect on their reproductive potential, preoviposition period and the inter-egg mass interval laid by the beetle. This protease inhibitor is potentially valuable for plant resistance against this insect. Similarly, Duan *et al.* (1996) who introduced the potato proteinase inhibitor II (*pin II*) gene into several Japonica rice varieties and regenerated a large number of transgenic rice plants and found that introduced *pin II* gene was stably inherited in the second, third and fourth generations. Several homozygous transgenic lines were obtained. Bioassay for insect resistance on the 5th generation transgenic rice plants with 2nd instar larvae of rice pink stem borer showed that transgenic rice plants had increased resistance to major rice insect pest, pink stem borer (*Sesamia inferens*). Larvae in the transgenic rice plants

showed little weight increase and stayed at the 2nd-3rd instar stage while larvae in the control plants showed 3 to 4-fold weight increase and developed to the 4th and 5th instar stage during the five-weeks of observation period.

Mochizuki *et al.* (1999) introduced a synthetic gene, *mwti1b*, coding for a winged bean trypsin inhibitor WTI-1B and expressed in rice plants, *O. sativa*. Protein extracts from transgenic rice plants expressing the trypsin inhibitor, inhibited the gut proteases of larvae of rice stem borer (*Chilo suppressalis*) *in vitro*. Growth of larvae fed on leaves of transgenic lines was reduced, compared to that of larvae fed on control rice plants. Similar results were obtained by Yeh *et al.* (1997) on tobacco cut worm, *Spodoptera litura* (F.), using sweet potato trypsin inhibitor gene in tobacco plants.

Orozco-Cardenas *et al.* (1993) reported that the expression of an antisense prosystemin gene in transgenic tomato decreased the ability of the plants to produce PIs and consequently reduced resistance towards *Manduca sexta* larvae. Bolter and Jongsma (1995), Bown *et al.* (1997), Broadway (1996) and Jongsma *et al.* (1995) provided the important results that insects can adapt to the ingestion of PIs. Some Lepidoptera and Coleoptera can over express the existing gut proteases, or induce the production of new types that are insensitive to the introduced PIs and overcome the deleterious effect of PI ingestion. Insects possess a complex pool of proteases and to avoid adaptation, the major proteases

must be inhibited. This could be achieved by expressing PIs of different types and or by improving the affinity of the introduced PI for the target insect proteases.

Consequently, Reeck *et al.* (1997) have realized from their studies that an individual proteinase inhibitor is not going to be universally useful as an insect growth retardant. The ability of individual insects in some species to adapt physiologically to the presence of inhibitors in their diets indicates that inhibitors may simply improve a stress on insects. To have a strong effect on insect growth and development, this stress should probably be applied in combination with stresses from products of other transgenes. One useful strategy of combined transgenes may be to use genes that encode inhibitors of more than one mechanistic class of proteinase. Tissue-specific expression or wound-inducible expression will obviously be highly desirable in some applications.

CHAPTER – III

MATERIALS AND METHODS

The studies entitled “Response of transgenic rice plants to rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and stem borer, *Scirpophaga incertulas* (Walker)”, were conducted in the Department of Biotechnology and Molecular Biology, and Department of Entomology, Chaudhary Charan Singh Haryana Agricultural University, (CCS HAU), during rice season of 2001 and 2002.

3.1 Materials Required

3.1.1 Test Insects

Adult moths of *C. medinalis* and *S. incertulas* were collected from paddy fields of Kaithal and Karnal districts of Haryana during paddy season because rice is the only cultivated host plant of *S. incertulas*, whereas *C. medinalis* has been reported to occur on maize and sorghum (Dale, 1994). Moths were fed on 10 per cent honey solution, and allowed to mate and oviposit in glass jars/oviposition cages containing rice plants. The egg masses laid by stem borer and leaffolder were collected from the plants and were transferred to vials. The neonate larvae from the eggs were used for bioassay.

3.1.2 Plants

Seeds of transgenic plants containing *pin II* gene were obtained from Department of Biotechnology and Molecular Biology, CCS HAU, Hisar.

Varieties	Transgenic lines
TNG 67 (Japonica variety)	T-TNG-64 T-TNG-61 T-TNG-46b T-TNG-26
Taraori Basmati	T-TB-95 T-TB-82 T-TB-57 T-TB-25
Pusa Basmati	PB-18-J1 PB-19-J1

were the lines established after transgenic on the basis of southern hybridization and northern hybridization (amplification of *pin II* protein) gene copy number etc.

TNG 67, Taraori Basmati and Pusa Basmati were used as the control plants for their transgenic lines separately.

3.1.3 Other Materials

Glass jars, insect rearing cages (Fig. 1), oviposition cages (Fig. 2), earthen pots, galvanized iron trays (large and small), nylon net, petridishes, glass vials etc. were arranged from the Department of Entomology of CCS HAU, Hisar.



Fig. 1: *Insect rearing cage*



Fig. 2: *Oviposition cage*



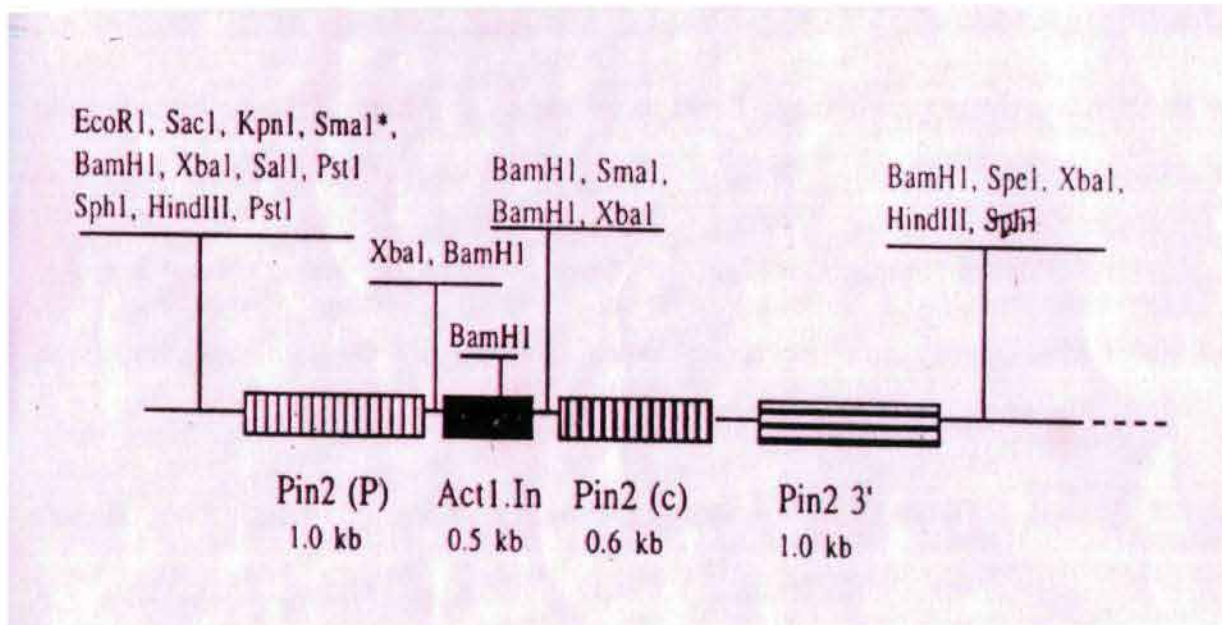
Fig. 3: Greenhouse used for transgenic studies



Fig. 4: A view of transgenic rice plants in the greenhouse

Fig. 5: Restriction map of plasmid RKJ1

RKJ1 (pCAMBIA 1200 + Pin2 P-Act1 In-Pin2 C-Pin 2 3')



Restriction map of RKJ1 (in pCAMBIA 1200)

Unique sites:	NIL
Plasmid Size:	RKJ1 -12.2 kb
Vector:	pCAMBIA 1200 for RKJ1
Agrobacterium strain:	LBA 4404
Selection agent:	Chloramphenicol 30 mg/l for <i>E. coli</i> Hygromycin 50 mg/l for rice
Promotor:	pin II wound inducible
Pin 2 (C):	Potato protease inhibitor 2 gene

3.1.4 Location of Experiment

The plants were grown in a containment greenhouse (Fig. 3 & 4) approved for growth of transgenic plants maintained at $25\pm 3^{\circ}\text{C}$, 70-95% RH and a photoperiod of 12:12 (L:D) h. The plants in all experiments were arranged in a Complete Randomized Design (CRD). For experiments using whole plants, the infested plants were also maintained in the containment greenhouse. For experiments using isolated leaves or stem pieces cut from plants, petridishes or glass vials containing the plant parts were placed in an air-conditioned room maintained at $25\pm 3^{\circ}\text{C}$, 70-95% RH and a photoperiod of 12:12 (L:D) h.

3.2 Experimental Methods

3.2.1 Evaluation of transgenic rice plants for *pin II* gene (potato proteinase inhibitor-II) by PCR (Polymerase Chain Reaction) analysis

The transgenic plants of rice were produced by *Agrobacterium* gene transformation method in Japonica cultivar, Taraori Basmati and Pusa basmati by the Department of Biotechnology and Molecular Biology, CCS HAU, Hisar. Plasmid RKJ1 containing the cloned *pin II* gene derived by wound inducible *pin II* promoter, was used for genetic transformation by them (Fig. 5).

The seeds (5-6) each of transgenic rice plants of T₁ (second) generation and T₂ (third) generation (A plant derived from tissue culture of transformed embryogenic callus is referred to as a T₀ plants, thus T₂ plants are the third plant generation after transformation) were sown in earthen pots (during the respective

years 2001 and 2002) of 4 kg capacity (20 cm high and 17 cm diameter) which were filled with soil and placed on cemented slab in greenhouse and saturated with water, two days before sowing the seeds. The pots were labelled randomly with aluminium tags. Fertilizer was applied on soil test basis in three splits before sowing, and 20 day intervals in pots. When the plants were one month old, the presence of potato hygromycin resistant gene and proteinase inhibitor-II (*pin II*) of transgenes was confirmed by Polymerase Chain Reaction (PCR) analysis as under:

DNA Isolation and Purification

DNA isolation was done by C-TAB extraction mini prep (Saghai-Marooif *et al.*, 1984) method using the leaf tissues. RNA was removed by treatment with 50 µg RNAs/10 µl DNA, with an incubation at 37°C for 30 min. DNA again extracted with equal volume of ice-cold chloroform: isoamyl alcohol and centrifuged at 12,000 rpm for 10 min. Top DNA containing solution was transferred to fresh tube and DNA precipitated by adding equal volume of pre-chilled isopropanol followed by ethanol washing.

PCR Amplification

PCR amplification was carried out using DNA samples isolated from leaf tissues of putative transgenic rice plants. A suitable pair of gene specific oligonucleotides was used for amplification of a DNA sequence of the useful gene. As an internal control, a set of primers, which amplify 304 bp product by PCR from Act 1 promoter region, was used. PCR assays were performed using PTC-100™ 9V thermocycler (MJ research, Inc., USA) and Taq ampligold DNA polymerase

(Promega, Inc., USA) with the following cycle parameters: 30 sec at 94°C, 45 sec at 62°C and 45 sec at 72°C. After 40 cycles, the PCR products were separated on a 1.5 per cent agarose gel and stained with ethidium bromide. The transgenic plants were identified with specific band as compared to control. The plants with negative response were removed from the pots.

3.2.2 Evaluation of transgenic rice plants for response against rice leaffolder, *C. medinalis* and stem borer, *S. incertulas*

To see the effect of plant growth stage on insect resistance, plants of the T2 generation at vegetative and earhead stage were evaluated against neonates of *C. medinalis* and *S. incertulas*.

Leaf-section/cut-leaf bioassay with *C. medinalis* and *S. incertulas* during vegetative stage of plants

Two small pieces (6-7 cm long) were cut from two different leaves close to the flag-leaves of two tillers from each of the transgenic line and control plants. Each leaf section was kept in petridishes (100 mm x 15 mm) lined with filter paper moistened with distilled water containing 100 ppm benzimidazol to prevent fungal contamination. There were five replications for each insect and for every transgenic line and control plants. The petridishes were arranged on shelves in a Complete Randomized Design. Each cut end was covered with a small piece of filter paper or cotton moistened with the benzimidazol solution. Five neonate larvae (0-12 h old) of *C. medinalis* and *S. incertulas* on each leaf section were transferred into petridishes separately. The larval mortality and the leaf area

consumed were recorded for four days. The leaf sections were renewed if required following Ye *et al.* (2000) and Alinia *et al.* (2000).

Cut-stem bioassay with *S. incertulas* during earhead (flowering) stage of plants

Two 6-7 cm stem pieces cut from tiller of transgenic plants were placed in petridishes lined with moist filter paper. Transgenic line and control plants were arranged in Complete Randomized Design and there were five replications for each. Each stem piece was infested with five Ist instar larvae of *S. incertulas*. The stems were dissected after four days and the number of dead and live larvae were recorded as per method of Alinia *et al.* (2000).

Whole plant bioassay during vegetative stage of plant with *C. medinalis* and *S. incertulas*

Ten (Ist instar) neonate larvae of *C. medinalis* and *S. incertulas* were placed for each replication of transgenic line/transgenic plant separately. Equal number of plants were kept in each pot. The potted plants were arranged in Complete Randomized Design in aluminium tray (180 cm x 75 cm x 15 cm) filled with water, with five replications for each insect of every transgenic line and one control plant. These plants were 3 month old and still at the vegetative stage because of photoperiod sensitivity. Each pot or replication was covered individually with a cage (85 cm x 36 cm diameter) for stem borer and in a common cage condition (180 cm x 75 cm x 15 cm) for leaffolder to prevent the

larvae from escaping. The extent of damage was scored by using Standard Evaluation System for rice (Anonymous, 1988).

For rice leaffolder, the transgenic plants were evaluated for leaf damage at 16 days after infestation. The damage grade was first determined for each leaf as given below:

Grade	Damage
0	None
1	Upto 1/3 of leaf area scraped
2	>1/3 to 1/2 of leaf area scraped
3	>1/2 of leaf area scraped

Thereafter, damage rating (R) for each entry was worked out in the following manner:

$$\text{Damage rating (R)} = \frac{\left(\frac{(\text{No. of leaves with damage grade of } 1 \times 100)1}{\text{Total no. of leaves observed}} + \frac{(\text{No. of leaves with damage grade } 2 \times 100)2}{\text{Total no. of leaves observed}} + \frac{(\text{No. of leaves with damage grade of } 3 \times 100)3}{\text{Total no. of leaves observed}} \right)}{6}$$

In the next step, adjusted damage rating (D) was calculated and level of resistance was determined in 0-9 scale as shown below:

$$\text{Adjusted damage rating (D)} = \frac{\text{Damage rating (R) of test entry}}{\text{Damage rating (R) of susceptible check}} \times 100$$

Scale	% adjusted damage rating
0	0
1	1-10
3	11-30
5	31-50
7	51-75
9	>75

For stem borer, the transgenic plants were evaluated for damage score after one month (30 days).

Percentage of dead hearts was worked out for each entry

$$\% \text{ dead hearts} = \frac{\text{No. of dead hearts}}{\text{Total no. of tillers observed}} \times 100$$

Per cent corrected dead hearts was worked out by the following formula:

$$D = \frac{\% \text{ dead hearts in test entry}}{\% \text{ dead hearts in susceptible check}} \times 100$$

Corrected per cent dead hearts was converted to a 0-9 scale as detailed below:

Scale	% dead hearts (D)
0	None
1	1-20
3	21-40
5	41-60
7	61-80
9	81-100

Whole plant bioassay with *S. incertulas* during earhead stage of plants

The method used was the same as given earlier. The 1st instar larvae of *S. incertulas* were released on transgenic plants during earhead stage of plants. After 14 days, white heads were counted, plants were dissected and the number of dead and live larvae were recorded. Weight and dimension of live larvae were recorded as suggested by Heinrichs *et al.* (1985).

3.2.3 To study the biology of rice leaffolder on transgenic rice plants

Three month old plants of transgenic lines in earthen pots of 4 kg capacity (20 cm x 17 cm diameter) at the rate of 3 plants per pot were infested with 10 neonate larvae. The larvae were placed near the auricles of the youngest leaf with the help of fine camel hair brush. One pot represented one replication and there were five replications for each transgenic line. Each pot was covered individually with a nylon cage (85 cm x 36 cm diameter). The observations on various biological parameters viz., larval survival, larval period, larval weight, per cent pupation, pupal period, pupal weight and moth emergence were recorded as suggested by Heinrichs *et al.* (1985).

3.2.4 To establish the mechanism of resistance in transgenic rice plants against rice leaffolder and stem borer

3.2.4.1 Rice leaffolder larvae feeding on transgenic rice plants

In this experiment, the pots were arranged in a Complete Randomized Design (CRD) in aluminium tray filled with water. In all, there were five

replications of each transgenic plant. Each replication was covered with a nylon net cage. Plants (3 month old) were infested individually with one 4th instar larva per plant under a common cage. The 4th instar larva was selected because of their aggressive feeding habit (Graf *et al.*, 1992). Two days after infestation, larvae were removed and leaf area damaged (scrapped) was measured with mm² graph paper (Heinrichs *et al.*, 1985; Anonymous, 1988).

3.2.4.2 Rice leaffolder and stem borer oviposition on transgenic plants

In this experiment, the arrangement of pots was similar as already mentioned. Three day old adults of both insects were released at the rate of 5 pairs per pot in each cage separately. Each pot was covered individually by oviposition cage (85 cm x 36 cm diameter). The number of eggs or egg masses laid on different plants were counted after 48 h (Anonymous, 1988).

3.2.5 Statistical analysis of data

The data obtained under various experiments were tabulated and subjected to statistical analysis as per requirement. Data in percentage were subjected to angular transformation whereas numerical data on duration of developmental stages were transformed to square root transformation (\sqrt{n}) before subjecting to statistical analysis (Snedecor and Cochran, 1968).

CHAPTER – IV

RESULTS

The response of rice transgenic lines containing serine type potato proteinase inhibitor gene was studied under green house conditions against rice insect-pests after confirming the presence of gene by polymerase chain reaction analysis (PCR). These transgenic lines were produced efficiently by Bhutani (2001) in the Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar. In this transformation, *pin II* gene coding for potato proteinase inhibitor was introduced into rice plants by an agrobacterium-mediated method using a hygromycin resistance gene, as selectable marker into the binary vector (pCAMBIA1200) construct (RKJ1) plasmid. In this construct, the expression of the potato proteinase inhibitor II gene (*pin II*) is regulated by its own promoter and 3' terminator sequence. The first intron of rice actin 1 gene was lying between *pin II* promoter and the *pin II* coding region. The previous studies (Xu *et al.*, 1996) have demonstrated that the combination of the *pin II* promoter and the act 1 intron confers high level wound-inducible expression of foreign genes in transgenic rice plants. The findings pertaining to Polymerase Chain Reaction analysis and response of transgenic lines to leafhopper

Cnaphalocrocis medinalis (Guenee) and stem borer *Scirpophaga incertulas* (Walker) have been given in this chapter.

4.1 Evaluation of transgenic rice plants for *pin II* gene (potato proteinase inhibitor II) by PCR (Polymerase Chain Reaction) analysis

The transgenic nature of rice plants of T₁ and T₂ generations (2nd and 3rd) generations of transgenic rice plants during the year 2001 and 2002 was confirmed by an improved PCR analysis procedure. Two sets of primers were used to amplify two DNA fragments: (i) a 304 bp size fragment from the rice actin 1 gene present in all types of rice varieties, and (ii) a 490 bp DNA fragment from *pin II* gene cassette. The record of PCR analysis is given in Table 1 and Table 2. The presence of both bands, one for rice actin 1 fragment (304 bp fragment) and another for *pin II* fragment (490 bp fragment) confirmed the transgenic nature of plants (Fig. 6 & 7). The segregation ratio between transgenic and non-transgenic plants was improved in T₂ generation of transgenic rice plants than T₁ generation. During the year 2001 rice season, 88 plants among 170 and in 2002 rice season, 155 plants among 217 hygromycin resistant rice lines were found to be transgenic for the *pin II* gene.

CONFIRMATION OF THE TRANSGENIC PLANTS BY PCR

Fig. 6

Lane 1 & 26	:	Eco R ₁ + Hind III digested λ DNA
Lane 2, 10, 14, 15, 16, 18, 19, 20, 21	:	Blank (No amplification was observed in few samples which was repeated again. Then it again amplified).
Lane 3, 4, 5, 6, 7, 8, 9, 12 & 13	:	Transformed plants showing amplified pin II fragment (490 bp)
Lane 11, 17, 22 & 23	:	Untransformed plants.
Lane 24	:	Plasmid DNA
Lane 25	:	Negative control.

Fig. 7

Lane 1	:	Eco R ₁ + Hind III digested λ DNA
Lane 3, 4, 5, 6, 7 & 8	:	Transformed plants showing amplified pin II fragment (490 bp)
Lane 9	:	Plasmid DNA
Lane 2 & 10	:	Blank

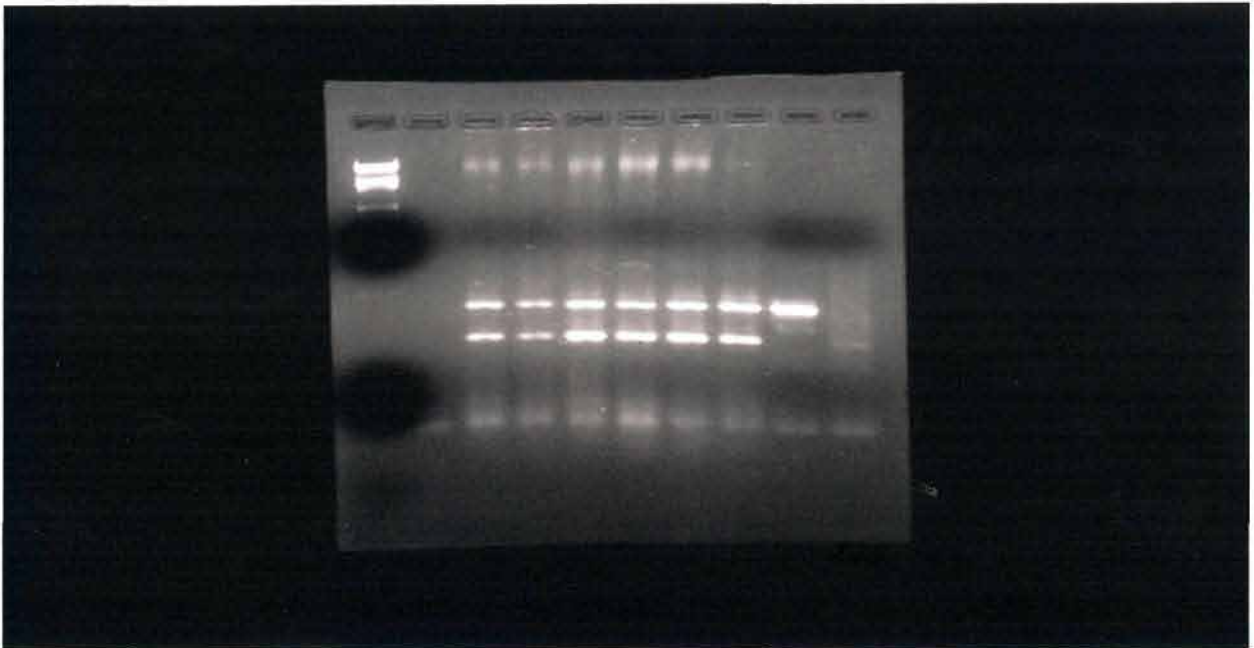
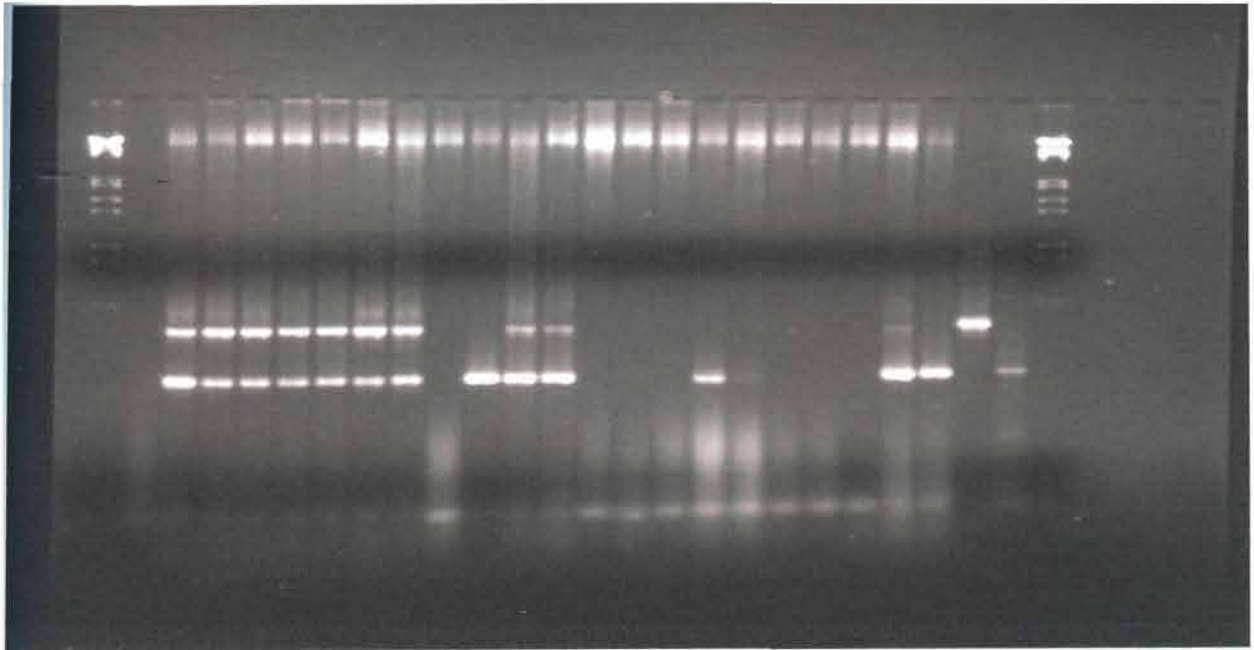


Fig. 6 & 7: Polymerase Chain Reaction (PCR) analysis of transgenic rice plants containing *pin II* gene

Table 1. Polymerase Chain Reaction (PCR) analysis of transgenic rice plants of T₁* (2nd) generation (2001) carrying *pin II* gene

Name of variety	Transgenic line	No. of T ₁ plants tested	PCR analysis		Ratio (positive: negative)
			Positive	Negative	
TNG 67 (Japonica variety)	T-TNG-64	14	7	7	1:1
	T-TNG-61	18	8	10	0.8:1
	T-TNG-46b	16	9	7	1.3:1
	T-TNG-26	18	10	8	1.2:1
Taraori basmati	T-TB-95	15	7	8	0.9:1
	T-TB-82	17	8	9	0.9:1
	T-TB-57	18	9	9	1:1
	T-TB-25	20	12	8	1.5:1
Pusa basmati	PB-18-J1	16	9	7	1.3:1
	PB-19-J1	18	9	9	1:1

*Seed harvested from first generation transgenic rice plants (T₀) were used in the above study.

Table 2. Polymerase Chain Reaction (PCR) analysis of transgenic rice plants of T₂* (3rd generation (2002) carrying *pin II* gene

Name of variety	Transgenic line	No. of T ₂ plants tested	PCR analysis		Ratio (positive: negative)
			Positive	Negative	
TNG 67 (Japonica variety)	T-TNG-64	28	19	9	2.1:1
	T-TNG-61	17	13	4	3.2:1
	T-TNG-46b	19	14	5	2.8:1
	T-TNG-26	28	20	8	2.5:1
Taraori basmati	T-TB-95	18	11	7	1.5:1
	T-TB-82	27	18	9	2:1
	T-TB-57	31	26	5	5.2:1
	T-TB-25	19	13	6	2.2:1
Pusa basmati	PB-18-J1	15	10	5	2:1
	PB-19-J1	15	11	4	2.7:1

*Seed harvested from the PCR positive T₁ plants were used to raise T₂ generation in the above study.

4.2 Evaluation of transgenic rice plants for response against rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and rice stem borer, *Scirpophaga incertulas* (Walker)

4.2.1 Response of transgenic rice plants using a leaf-section/cut-leaf bioassay

Cut-leaf bioassay with *C. medinalis* during vegetative stage of plants

Neonate larvae were released on the small pieces of leaves of growing plants in petridishes for four days. The per cent mortality was recorded on 2nd and 4th day and total feeding leaf area in mm² was also recorded (Table 3). The mortality of Ist instar larvae of rice leaffolder on 2nd day on transgenic plants was 52.50 to 67.50 per cent and on 4th day it increased to 80.00 to 95.00 per cent. On some transgenic lines (T-TNG-61, T-TNG-64, T-TNG-46b and T-TB-82) the per cent larval mortality was more than 85.00 after four days of feeding on cut leaf pieces.

With respect to leaf area damage/feeding, Taraori Basmati was most preferred for feeding by rice leaffolder and the leaf area consumed was 23.00 mm². Leaf area scraped was very less on transgenic lines, T-TNG-46b and T-TNG-61 (4.12 and 4.50 mm²). There was a significant difference in leaf area eaten between transgenic and non-transgenic plants within four day of release. The highest larval mortality (95.00%) and lowest leaf feeding area (4.12 mm²) by rice leaffolder larva were observed on transgenic line T-TNG-46b of TNG67.

Table 3. The cut-leaf bioassay with *Cnaphalocrocis medinalis* (Guenee) during vegetative stage of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	Larval mortality (%)		Leaf area eaten after four days (mm ²)
		2 nd day	4 th day	
1.	T-TNG-64	57.50 (49.31) ^{bcd}	87.50 (72.09) ^{bcd}	11.37 ^{ef}
2.	T-TNG-61	65.00 (53.76) ^{bcd}	87.50 (72.09) ^{bcd}	4.50 ^{ab}
3.	T-TNG-46b	67.50 (55.48) ^{cde}	95.00 (83.35) ^d	4.12 ^a
4.	T-TNG-26	57.50 (49.31) ^{bcd}	82.50 (68.40) ^{bcd}	8.50 ^{cde}
5.	TNG67(C)	4.00 (11.54) ^a	4.00 (11.54) ^a	19.12 ^g
6.	T-TB-95	55.00 (47.86) ^{bc}	85.00 (67.47) ^{bc}	11.12 ^{ef}
7.	T-TB-82	72.50 (58.43) ^e	92.50 (81.69) ^{cd}	5.62 ^{abc}
8.	T-TB-57	65.00 (53.76) ^{bcd}	85.00 (67.47) ^{bc}	7.85 ^{cd}
9.	T-TB-25	52.50 (46.42) ^b	80.00 (63.78) ^b	7.12 ^{bcd}
10.	Taraori Basmati (C)	4.00 (11.54) ^a	4.00 (11.54) ^a	23.00 ^h
11.	PB-18-J1	56.50 (49.37) ^{bcd}	87.50 (72.09) ^{bcd}	9.50 ^{def}
12.	PB-19-J1	52.50 (46.42) ^b	82.50 (68.40) ^{bcd}	12.25 ^f
13.	Pusa Basmati (C)	4.00 (11.54) ^a	4.00 (11.54) ^a	18.62 ^g
	Mean	47.19	67.46	10.98
	SE(m)	3.94	4.35	1.04
	CD (P=0.05)	(8.10)	(15.26)	2.98

The values in parenthesis are angular transformed values.

Data based on 5 replications and each replicate consists of two stem pieces in one petridish with 10 Ist instar larvae.

C – control.

Cut-leaf bioassay with *S. incertulas* during vegetative stage of plants

In this experiment also, the Ist instar larvae were released on leaf pieces in petridishes and larval mortality on 2nd and 4th day and total feeding area were observed (Table 4). The mortality of rice stem borer larvae was significantly higher after two and four days in transgenic rice plants which were tested for insect bioassay than in non-transformed plants (control plants). The highest mortality was observed in transgenic line T-TNG-61 of TNG67 (Japonica cultivar) on 2nd day and the mortality of larvae was above 55.00 per cent and above 85.00 per cent after four days in all transgenic rice plants than control plants. The observations on leaf area damage after four days of infestation revealed that rice variety Taraori Basmati (non-transgenic) was most preferred for feeding by *S. incertulas* on which 49.62 mm² leaf area was scraped followed by non-transgenic Pusa Basmati and Japonica cultivar. The larval feeding on all transgenic plants was significantly less than all non-transgenic plants (Table 4). The lowest feeding area was observed on transgenic plant no. T-TNG-61 of TNG67 (9.37 mm²) followed by plant No. T-TB-57, T-TB-95 and T-TB-82 of Taraori Basmati (9.62, 9.75 and 9.75 mm²).

Cut-stem bioassay with *S. incertulas* during earhead stage of plants

Small cut-stem pieces were used for feeding of neonate larvae of stem borer. After four days, the per cent survival was recorded (Table 5). At the earhead stage, neonate survival was higher on transgenic plants as compared to

Table 4. The cut leaf bioassay for *Scirpophaga incertulas* (Walker) during vegetative stage of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	Larval mortality (%)		Leaf area eaten after four days (mm ²)
		2 nd day	4 th day	
1.	T-TNG-64	60.00 (50.75) ^f	87.50 (69.50) ^b	13.75 ^a
2.	T-TNG-61	80.00 (63.78) ^b	95.00 (83.35) ^b	9.37 ^a
3.	T-TNG-46b	60.00 (50.75) ^{bc}	85.00 (67.47) ^b	14.50 ^{ab}
4.	T-TNG-26	62.50 (52.25) ^b	92.50 (81.69) ^b	25.25 ^b
5.	TNG67(C)	2.00 (8.13) ^a	2.00 (8.13) ^a	35.12 ^c
6.	T-TB-95	60.00 (50.75) ^b	92.50 (81.69) ^b	9.75 ^a
7.	T-TB-82	60.00 (50.75) ^b	92.50 (81.69) ^b	9.75 ^a
8.	T-TB-57	67.50 (55.24) ^{bcde}	92.50 (81.69) ^b	9.62 ^a
9.	T-TB-25	55.00 (47.86) ^b	85.00 (67.47) ^b	16.50 ^{ab}
10.	Taraori Basmati (C)	4.00 (11.54) ^a	4.00 (11.54) ^a	49.62 ^d
11.	PB-18-J1	62.50 (52.25) ^{bc}	87.50 (69.50) ^b	13.25 ^a
12.	PB-19-J1	65.00 (53.76) ^{bcd}	92.50 (81.69) ^b	18.50 ^{ab}
13.	Pusa Basmati (C)	4.00 (11.54) ^a	4.00 (11.54) ^a	48.87 ^d
	Mean	49.42	70.19	21.06
	SE(m)	3.49	4.46	3.81
	CD (P=0.05)	(8.44)	(16.84)	10.94

The values in parenthesis are angular transformed values.

Data based on 5 replications and each replicate consists of two leaf pieces in one petridish with 10 Ist instar larvae.

C – control.

Table 5. The cut-stem bioassay for *Scirpophaga incertulas* (Walker) during reproductive stage (after ear emergence) of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	Larval survival (%)
1.	T-TNG-64	26.663 (30.959) ^{ab}
2.	T-TNG-61	31.110 (33.673) ^{ab}
3.	T-TNG-46b	26.440 (29.568) ^{ab}
4.	T-TNG-26	37.773 (37.786) ^{bc}
5.	TNG67(C)	82.220 (65.765) ^d
6.	T-TB-95	19.997 (26.345) ^a
7.	T-TB-82	19.997 (26.345) ^a
8.	T-TB-57	22.220 (27.736) ^a
9.	T-TB-25	37.773 (37.786) ^{bc}
10.	Taraori Basmati (C)	86.663 (68.987) ^d
11.	PB-18-J1	28.887 (32.350) ^{ab}
12.	PB-19-J1	48.883 (44.342) ^c
13.	Pusa Basmati (C)	86.663 (68.987) ^d
	Mean	42.71
	SE(m)	4.44
	CD (P=0.05)	(9.25)

The values in parenthesis are angular transformed values.

Data based on 5 replications and each replicate consists of two stem pieces in one petridish with 10 1st instar larvae.

C – control.

vegetative stage of these plants, but it was significantly lower than on non-transgenic plants. The survival of 1st instar larvae as recorded after four days was approximately 49 per-cent in PB-19-J1 (transgenic of Pusa Basmati), 38 per cent in transgenic plant no. T-TNG-26 and T-TB-25 (transgenic of TNG67 and Taraori Basmati), 20 to 31 per cent in T-TB-95, T-TB-82, T-TB-57, T-TNG-64, T-TNG-46b, PB-18-J1 and T-TNG-61 transgenic plants. The larval survival was more at earhead stage as compared to at vegetative stage but the development of surviving larvae on transgenic plants proceeded upto 2nd-3rd instar and the larvae were lighter in weight as compared to control.

4.2.2 Response of transgenic rice plants using whole plant bioassay

The whole plant bioassay with *C. medinalis* at vegetative stage of plants

In this experiment, the observations were recorded on the basis of leaf damage after 16 days of infestation. Out of ten transgenic plant nos. tested against rice leaffolder, seven (T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82, T-TB-57 and T-TB-25) were found resistant with a damage score of 3 (Table 6, Fig. 8 & 9). The other three were found moderately resistant with a damage score of 5. The damage rating on control plants was 3 to 4-fold more than transgenic plants. Japonica variety (TNG67) and Basmati rice (control plants) were more preferred by *C. medinalis*.



Fig. 8

TNG67

T-TNG-61



Fig. 9

T-TNG-64

TNG67

Fig. 8 & 9: Resistance expression in transgenics (*pin II* gene) to rice leaffolder (whole plant bioassay)

Table 6. The whole plant bioassay for *Cnaphalocrocis medinalis* (Guenee) at vegetative stage of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	Damage rating (R) (%)	Adjusted damage rating D(%)	Grade*
1.	T-TNG-64	4.98	20.17	3
2.	T-TNG-61	5.93	24.02	3
3.	T-TNG-46b	3.90	15.79	3
4.	T-TNG-26	9.26	39.62	5
5.	TNG67(C)	24.69	100.00	9
6.	T-TB-95	3.75	16.25	3
7.	T-TB-82	3.10	13.26	3
8.	T-TB-57	4.03	17.24	3
9.	T-TB-25	4.79	19.40	3
10.	Taraori Basmati (C)	23.37	100.00	9
11.	PB-18-J1	7.29	33.55	5
12.	PB-19-J1	7.44	34.24	5
13.	Pusa Basmati (C)	21.73	100.00	9

Data based on 5 replications. Each replicate consists of two PCR positive T₂ plants in one pot with 10 1st instar larvae.

C – control.

*Standard evaluation system for rice (Anonymous, 1988). Grading based on 0-9 scale.

The whole plant bioassay for stem borer during vegetative and earhead stage of plants

In the whole plant bioassay at vegetative stage of plants, the number of dead hearts were counted after one month. Transgenic lines viz., T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-82, T-TB-95 and PB-18-J1 (Table 7) were found resistant with a damage rating of 38.56 to 40.41 per cent and a damage score of 3 (Fig. 10 & 11). However, four transgenic lines viz., T-TNG-26, T-TB-57, T-TB-25 and PB-19-J1 were found moderately resistant with a damage rating from 46.93 to 50.07 per cent and a damage score of 5. During vegetative stage of plants, the non-transgenic (control) plants were found highly infested by stem borer larvae. Growth of the larvae fed on transgenic plants was reduced as compared to the larvae fed on control rice plants (Fig. 12 & 13). The larvae reared on transgenic plants showed restricted development and remained in the 2nd or 3rd instar stage while larvae on control plants showed 2 to 3-fold weight increase and developed upto 4th or 5th instar stage.

Approximately 85.00 per cent of tillers in control plants showed susceptibility with white head symptoms whereas in transgenic plants, response varied to give the status of resistant and moderately resistant.

During earhead stage (after ear emergence), the plants were dissected after 14 days. Only three transgenic lines (T-TNG-64, T-TNG-61 and T-TB-82) showed resistance with a damage grade of 3, other seven transgenic plant no.



Fig. 10

Taraori Basmati

T-TB-82



Fig. 11

PB-18-J1

Pusa Basmati

Fig. 10 & 11: Resistance expression in transgenics (*pin II* gene) to stem borer (whole plant bioassay)



Fig. 12: Stem borer larvae fed on transgenics (a) and non-transgenics (b)



Fig. 13: Stem borer larva feeding inside the stem

showed moderate resistance with a damage grade of 5 (Table 8). After emergence of the earhead, damage by larvae was less and this may be due to decrease in the level of proteinase inhibitors. The survival (Table 9) in transgenic plants ranged from 33.33 to 43.33 per cent. More number of larvae survived but they were unhealthy and of restricted growth and died after 2 or 3 days. However, per cent larval survival on transgenic plants differed significantly from that on control plants. Weight of the larvae (Table 9) fed on transgenic plants expressing *pin II* gene was significantly low than those fed on control plants. The lowest larval weight was observed on T-TNG-64 transgenic line (9.00 mg) which was at par with T-TB-82 (9.20 mg), T-TNG-46b (10.00 mg) and T-TB-95 (10.40 mg). Similarly the dimension of larvae (mm^2) was also significantly less as compared to control plants.

4.3 Biology of rice leaffolder on transgenic rice plants

The various biological parameters viz., larval survival, growth and development, larval weight, per cent pupation, pupal period, pupal weight and moth emergence were recorded on transgenic lines to assess antibiosis mechanism of resistance. The larval survival ranged from 30.00 to 48.00 per cent on transgenic plants designated as resistant and moderately resistant in whole plant bioassay (Table 10). Larval survival on all transgenic plants differed significantly from that on susceptible control plants, TNG67, Taraori Basmati and Pusa Basmati (84.00 to 92.00%).

Table 7. The whole plant bioassay for *Scirpophaga incertulas* (Walker) during vegetative stage of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	Dead heart (%)	Adjusted damage rating (%)	Grade*
1.	T-TNG-64	31.39	38.72	3
2.	T-TNG-61	32.76	40.41	3
3.	T-TNG-46b	31.26	38.56	3
4.	T-TNG-26	46.93	57.89	5
5.	TNG67(C)	81.06	100.00	9
6.	T-TB-95	32.04	38.30	3
7.	T-TB-82	32.64	39.02	3
8.	T-TB-57	48.64	58.15	5
9.	T-TB-25	48.27	57.69	5
10.	Taraori Basmati (C)	83.64	100.00	9
11.	PB-18-J1	34.00	40.19	3
12.	PB-19-J1	50.07	59.19	5
13.	Pusa Basmati (C)	84.59	100.00	9

Data based on 5 replications. Each replicate consists of two PCR positive T₃ plants in one pot with 10 Ist instar larvae.

C – control.

*Standard evaluation system for rice (Anonymous, 1988). Grading based on 0-9 scale.

Table 8. The whole plant bioassay for *Scirpophaga incertulas* (Walker) during reproductive stage (after ear emergence) of transgenic rice plants containing *pin II* gene in T₂ generation

Sr. No.	Transgenic line	White ears (%)	Adjusted damage rating (%)	Grade*
1.	T-TNG-64	15.78	40.17	3
2.	T-TNG-61	12.53	31.90	3
3.	T-TNG-46b	16.55	42.13	5
4.	T-TNG-26	21.36	54.38	5
5.	TNG67(C)	39.28	100.00	9
6.	T-TB-95	20.39	41.67	5
7.	T-TB-82	11.49	23.49	3
8.	T-TB-57	22.48	45.96	5
9.	T-TB-25	25.32	51.76	5
10.	Taraori Basmati (C)	48.92	100.00	9
11.	PB-18-J1	21.15	47.56	5
12.	PB-19-J1	19.96	44.88	5
13.	Pusa Basmati (C)	44.47	100.00	9

Data based on 5 replications. Each replicate consists of two PCR positive T₃ plants in one pot with 10 Ist instar larvae.

C – control.

*Standard evaluation system for rice (Anonymous, 1988). Grading based on 0-9 scale.

Table 9. Development and survival of larvae of *Scirpophaga incertulas* (Walker) as assayed by whole plan bioassay after 14 days (after ear emergence) in *pin II* containing transgenic rice lines in T₂ generation

Sr. No.	Transgenic line	Larval survival (%) [*]	Larval weight (mg) [*]	Dimension of larvae (LxW) [*] (mm ²)
1.	T-TNG-64	36.00 (36.81) ^{ab}	9.00 ^a	4.90 ^a
2.	T-TNG-61	40.00 (39.17) ^b	11.40 ^{ab}	6.30 ^a
3.	T-TNG-46b	44.00 (41.52) ^b	10.00 ^a	6.50 ^a
4.	T-TNG-26	44.00(41.52) ^b	14.00 ^b	6.90 ^a
5.	TNG67(C)	80.00 (63.71) ^c	27.60 ^c	27.00 ^b
6.	T-TB-95	36.00 (36.81) ^{ab}	10.40 ^{ab}	4.00 ^a
7.	T-TB-82	30.00 (33.07) ^a	9.20 ^a	2.50 ^a
8.	T-TB-57	40.00 (39.17) ^b	14.00 ^b	4.20 ^a
9.	T-TB-25	42.00 (40.37) ^b	14.00 ^b	5.60 ^a
10.	Taraori Basmati (C)	92.00 (74.07) ^d	34.60 ^d	36.40 ^c
11.	PB-18-J1	36.00 (36.81) ^{ab}	11.20 ^{ab}	6.30 ^a
12.	PB-19-J1	36.00 (36.81) ^{ab}	14.20 ^b	9.00 ^a
13.	Pusa Basmati (C)	92.00 (74.07) ^d	30.40 ^c	32.40 ^{bc}
	Mean	49.85	16.15	11.6
	SE(m)	2.60	1.48	2.70
	CD (P=0.05)	(5.20)	4.20	7.60

The values in parentheses are angular transformed values.

Data based on 5 replications. Each replicate consists of two PCR positive T₂ plants in one pot with 10 Ist instar larvae.

C – control.

*Larval parameters based on average of 10 larvae.

Table 10. Effect of feeding transgenic rice leaf tissues containing *pin II* gene in T₂ generation on larval parameters of *Cnaphalocrocis medinalis* (Guenee)

Sr. No.	Transgenic line	Larval survival (%) [*]	Larval period (days) ^{**}	Larval weight (mg)
1.	T-TNG-64	32.00 (34.28) ^{ab}	27.00 (5.29) ^{cd}	12.40 ^{bc}
2.	T-TNG-61	34.00 (35.67) ^{ab}	27.40 (5.33) ^{cde}	12.00 ^{abc}
3.	T-TNG-46b	34.00 (35.67) ^{ab}	28.80 (5.46) ^{de}	13.00 ^{bc}
4.	T-TNG-26	46.00 (42.67) ^b	22.40 (4.83) ^b	17.00 ^d
5.	TNG67(C)	92.00 (81.69) ^d	17.20 (4.26) ^a	27.40 ^f
6.	T-TB-95	36.00 (36.87) ^{ab}	25.80 (5.18) ^c	11.00 ^{ab}
7.	T-TB-82	30.00 (32.47) ^a	29.40 (5.51) ^e	9.80 ^a
8.	T-TB-57	44.00 (41.47) ^{ab}	25.60 (5.16) ^c	13.00 ^{bc}
9.	T-TB-25	42.00 (40.32) ^{ab}	26.20 (5.21) ^c	13.40 ^c
10.	Taraori Basmati (C)	90.00 (80.99) ^d	17.80 (4.33) ^a	24.00 ^e
11.	PB-18-J1	40.00 (34.16) ^{ab}	22.20 (4.81) ^b	17.80 ^d
12.	PB-19-J1	48.00 (43.83) ^b	21.20 (4.71) ^b	17.80 ^d
13.	Pusa Basmati (C)	84.00 (66.42) ^c	18.80 (4.45) ^a	24.00 ^e
	Mean	50.15	26.09	16.35
	SE(m)	4.61	0.69	0.81
	CD (P=0.05)	(10.10)	(0.20)	2.30

Data based on 5 replications. Each replicate consists of two PCR positive T₂ plants in one pot with 10 1st instar larvae. Larval parameters based on average of 10 larvae.

*The values in parenthesis are angular transformed values.

** The values in parenthesis are square root transformed values.

C – control.

Likewise great variation in larval development among the transgenic plants was observed. The larval period (17.20 to 29.40 days) differed significantly among the test plants. Resistant transgenic plant nos. viz., T-TNG-64, T-TNG-61, T-TB-95, T-TB-82, T-TB-57 and T-TB-25 manifested more than 25.0 days of larval period as compared to control plants TNG67 (17.20 days), Taraori Basmati (17.80 days) and Pusa Basmati (18.80 days). However, the larval duration was intermediate (21.20 to 22.40 days) on transgenic lines, T-TNG-26, PB-18-J1 and PB-19-J1 (Table 10).

The weight of larvae on different transgenic plants ranged from 9.80 to 17.80 mg. The larval weight was poor (9.80 to 13.40 mg) when reared on T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82, T-TB-57 and T-TB-25 in comparison with susceptible checks TNG67 (27.40 mg), Taraori Basmati (24.00 mg) and Pusa Basmati (24.00 mg) (Fig. 14 & 15). Weight of the larvae raised on T-TNG-26, PB-18-J1 and PB-19-J1 transgenic plants was also significantly less (17.00 to 17.80 mg) than on control plants (Table 10).

The trend of per cent pupation was similar to larval survival on different transgenic plants. The number of larvae which underwent pupation was significantly low on all the transgenic plants tested as compared to non-transgenic plants. The minimum pupation was recorded on T-TNG-46b (10.00%) and maximum on T-TNG-26 (26.00%) in comparison with 84.00 to 92.00 per cent on



Fig. 14: Transgenic plants covered individually for biological studies



Fig. 15 Leaffolder larvae fed on transgenics (a) and non-transgenics (b)

Taraori Basmati, Pusa Basmati and TNG67, respectively (Table 11). However, the number of pupae on different transgenic plants was variable.

The mean pupal duration (Table 11) of rice leaffolder reared on different transgenic plants was also variable. The pupal period was on par with each other except PB-19-J1 (11.60 days), T-TG-26 (12.00 days) and PB-18-J1 (13.40 days). However, on all transgenic plants, pupal period was significantly longer than control plants (6.40 to 8.20 days).

The pupal weight on all transgenic plants was significantly lower (10.60 to 16.00 mg) than those reared on TNG67 (24.60 mg), Taraori Basmati (24.60 mg) and Pusa Basmati (22.00 mg). The lowest pupal weight was recorded on T-TB-95 (10.60) and T-TB-82 (10.80 mg). Moth emergence percentage based on initially released larvae differed significantly on all transgenic plants as compared to control plants.

4.4 Mechanisms of Resistance

4.4.1 Rice leaffolder larvae feeding on transgenic rice plants

The larval feeding on leaves of rice plants, a measure of relative susceptibility of cultivars was studied by infesting individual plant with one 4th-instar larva. The observation on leaf area damage after 48 h of infestation revealed that rice variety TNG67 was most preferred for feeding among the test plants, on which 435.20 mm² leaf area was scraped by the larvae. The larval feeding on transgenic plants of Pusa Basmati and one of Taraori Basmati (T-TB-

Table 11. Effect of feeding transgenic rice leaf tissues containing *pin II* gene in T₂ generation on pupal parameters of *Cnaphalocrocis medinalis* (Guenee)

Sr. No.	Transgenic line	Pupation (%) [*]	Pupal period (days) ^{**}	Pupal weight (mg)	Moth emergence (%) [*]
1.	T-TNG-64	16.00 (23.30) ^{abc}	15.60 (4.07) ^f	12.40 ^{abc}	14.00 (21.68) ^{abc}
2.	T-TNG-61	18.00 (24.63) ^{abc}	15.20 (4.02) ^{ef}	14.40 ^{bcde}	10.00 (16.37) ^{ab}
3.	T-TNG-46b	10.00 (16.37) ^a	15.80 (4.09) ^f	11.80 ^{ab}	6.00 (11.06) ^a
4.	T-TNG-26	26.00 (29.52) ^c	12.00 (3.60) ^{cd}	13.00 ^{abcd}	8.00 (14.74) ^{ab}
5.	TNG67(C)	92.00 (81.69) ^e	6.40 (2.71) ^a	24.60 ^c	90.00 (72.45) ^d
6.	T-TB-95	16.00 (23.30) ^{abc}	14.80 (3.97) ^{ef}	10.60 ^a	12.00 (17.99) ^{abc}
7.	T-TB-82	12.00 (20.05) ^{ab}	15.80 (4.09) ^f	10.80 ^a	10.00 (16.37) ^{ab}
8.	T-TB-57	26.00 (29.52) ^c	16.60 (4.18) ^f	15.60 ^{de}	20.00 (23.78) ^{bc}
9.	T-TB-25	20.00 (26.26) ^{bc}	16.80 (4.21) ^f	14.60 ^{cd}	24.00 (28.37) ^c
10.	Taraori Basmati (C)	90.00 (80.99) ^e	7.20 (2.85) ^a	24.60 ^c	88.00 (70.82) ^d
11.	PB-18-J1	24.00 (29.21) ^c	13.40 (3.79) ^{de}	15.80 ^d	16.00 (23.30) ^{bc}
12.	PB-19-J1	24.00 (29.21) ^c	11.60 (3.54) ^c	16.00 ^d	18.00 (24.93) ^{bc}
13.	Pusa Basmati (C)	84.00 (66.42) ^d	8.20 (3.03) ^b	22.00 ^e	84.00 (66.66) ^d
	Mean	35.23	13.03	15.86	30.77
	SE(m)	4.51	0.63	0.95	3.68
	CD (P=0.05)	(9.15)	(0.24)	2.72	(11.14)

Data based on 5 replications. Each replicate consists of two PCR positive T₂ plants in one pot with 10 1st instar larvae. Pupal parameters based on average of 10 pupae.

*The values in parenthesis are angular transformed values.

** The values in parenthesis are square root transformed values.

C – control.

Table 12. *Cnaphalocrocis medinalis* (Guenee) 4th instar larva feeding on transgenic rice plants containing *pin II* gene

Sr. No.	Transgenic line	Larval feeding after 48 h (mm ³)
1.	T-TNG-64	247.40 ^{ab}
2.	T-TNG-61	259.00 ^{ab}
3.	T-TNG-46b	242.00 ^a
4.	T-TNG-26	310.60 ^{bcd}
5.	TNG67(C)	435.20 ^e
6.	T-TB-95	294.60 ^{abcd}
7.	T-TB-82	235.60 ^a
8.	T-TB-57	275.60 ^{abc}
9.	T-TB-25	267.00 ^{ab}
10.	Taraori Basmati (C)	352.60 ^d
11.	PB-18-J1	294.00 ^{abcd}
12.	PB-19-J1	308.40 ^{bcd}
13.	Pusa Basmati (C)	335.20 ^{cd}
	Mean	276.70
	SE(m)	22.69
	CD (P=0.05)	64.59

Data based on 5 replications. One replicate consists of one 4th instar larva.

C – control.

Table 13. The oviposition of *Cnaphalocrocis medinalis* (Guenee) on transgenic rice plants containing *pin II* gene

Sr. No.	Transgenic line	No. of egg laid after 48 h
1.	T-TNG-64	51.60 (7.22) ^{acd}
2.	T-TNG-61	60.80 (7.84) ^d
3.	T-TNG-46b	58.40 (7.70) ^{cd}
4.	T-TNG-26	59.20 (7.75) ^{cd}
5.	TNG67(C)	59.80 (7.78) ^{cd}
6.	T-TB-95	43.40 (6.65) ^a
7.	T-TB-82	44.20 (6.72) ^a
8.	T-TB-57	42.60 (6.75) ^a
9.	T-TB-25	47.20 (6.57) ^a
10.	Taraori Basmati (C)	50.60 (7.17) ^{abc}
11.	PB-18-J1	47.60 (6.90) ^{ab}
12.	PB-19-J1	47.00 (6.85) ^a
13.	Pusa Basmati (C)	55.60 (7.52) ^{bcd}
	Mean	51.38
	SE(m)	3.37
	CD (P=0.05)	(0.66)

The values in parenthesis are square root transformation.

Data based on 5 replications. Each replication covered individually with a cage and in each replication five pairs of 3 day old adults were released.

C –control.

Table 14. The oviposition of *Scirpophaga incertulas* (Walker) on transgenic rice plants containing *pin II* gene

Sr. No.	Transgenic line	No. of egg masses per transgenic line after 48 h
1.	T-TNG-64	8.20
2.	T-TNG-61	7.60
3.	T-TNG-46b	8.60
4.	T-TNG-26	7.80
5.	TNG67(C)	9.20
6.	T-TB-95	8.20
7.	T-TB-82	7.00
8.	T-TB-57	7.60
9.	T-TB-25	8.20
10.	Taraori Basmati (C)	9.20
11.	PB-18-J1	8.40
12.	PB-19-J1	9.00
13.	Pusa Basmati (C)	9.20
	Mean	8.32
	SE(m)	0.83
	CD (P=0.05)	NS

Data based on 5 replications. Each replication covered individually with a cage and in each replication five pairs of 3 day old adults were released.

C – control.

95) did not differ significantly (Table 12). However, on other seven transgenic lines, leaf area damage was significantly less than control plants (non-transgenic plants).

4.4.2 Rice leaffolder and stem borer oviposition on transgenic plants

Oviposition of rice leaffolder for different rice test plants was observed under caged conditions in a no choice test. The cage arrangement was separate for rice leaffolder and stem borer.

The eggs were counted on individual plants after 48 h and the data are presented in Table 13 and 14. The adults of rice leaffolder and stem borer did not show distinct ovipositional differences among different transgenic plants and non-transgenic control plants i.e. no significant difference was observed with respect to oviposition on transgenic and non-transgenic plants.

CHAPTER – V

DISCUSSION

Rice is one of the most important crops for which efficient transformation/regeneration systems have been developed. Transgenic rice plants with improved agronomical traits have been produced by introducing foreign genes (Fujimoto *et al.*, 1993; Hayakawa *et al.*, 1992). The present studies were carried out on transgenic plants which contained a well-characterized serine type proteinase inhibitor gene from a dicot plant, potato into rice.

Results on the studies entitled “Response of transgenic rice plants to rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and stem borer, *Scirpophaga incertulas* (Walker)” are discussed in this chapter in view of work done by earlier workers.

5.1 Evaluation of transgenic rice plants for *pin II* gene by PCR analysis

The inheritance of the integrated *pin II*-containing gene cassette (transgene) in transgenic rice plants of second (T₁) and third (T₂) generations was investigated by Polymerase Chain Reaction (PCR) analysis. In the second generation (T₁), among 170 plants, 88 T₁ (51.76%) and in the third generation (T₂), among 217 plants, 155 T₂ (71.43%) contained the *pin II* (potato proteinase inhibitor II) gene. The

segregation ratio of transgenic: non-transgenic plants was increased (Table 1 and 2) during the successive generation as compared to their parent plants as observed by Duan *et al.* (1996) who recorded the increase in level of proteinase inhibitors in successive generation. They introduced the *pin II* gene into several Japonica rice varieties and found that introduced *pin II* gene was stably inherited in the second, third and fourth generations. These findings derive indirect support from the work of Alinia *et al.* (2000) who observed that the expression of Cry 1 Ab gene in line 827 was stable for seven generations after transformation.

5.2 Evaluation of transgenic rice plants for response against rice leaffolder and stem borer

5.2.1 Response of transgenic rice plants using a leaf/stem-section bioassay

Cut-leaf bioassay with *C. medinalis* during vegetative stage of plants

The per cent larval mortality was significantly higher and leaf feeding area was significantly less on all transgenic plants of rice which contained the *pin II* gene (Table 3) as compared to control plants. These observations confirm the defensive role of proteinase inhibitors as proposed by Reese (1983). According to him, proteinase inhibitors against insect herbivores work as anti-nutritional factors causing reduced digestion of dietary protein via direct inhibition of proteases. The transgenic lines viz., T-TNG-64, T-TNG-61, T-TNG-46 b, T-TB-95, T-TB-82, T-TB-57 and PB-18-J1 were responsible for above 85.00 per cent larval mortality and proved highly resistant as compared to other transgenic plants.

Cut-leaf bioassay with *S. incertulas* during vegetative stage of plants

The mortality of *S. incertulas* larvae was significantly higher after two and four days in transgenic plants while leaf area consumed was less than in non-transgenic rice plants. Consequently, very low levels of leaf damage (9.37 mm²) and highest mortality (95.00%) after four days was observed on transgenic plant No. 61 of TNG-57 (Table 4). The other five transgenic lines namely T-TNG-26, T-TB-95, T-TB-82, T-TB-57 and PB-19-J1 caused mortality around 90-92.50 per cent which was at par with that on T-TNG-61. The damaged leaf area was also at par among T-TNG-61, T-TB-95, T-TB-82 and T-TB-57 but was significantly different from other lines except T-TNG-64 and T-TB-25. The findings with respect to reduced damage due to *S. incertulas* and *C. medinalis* in transformed rice plants compared to non-transformed plants support the work of Duan *et al.* (1996), who reported significant differences between transgenic rice plants harbouring an introduced *pin II* gene and non-transformed plants in their response to infestation by rice pink stem borer and development of white head symptoms.

Cut-stem bioassay with *S. incertulas* during earhead stage of plants

To see the influence of plant growth stage on rice stem borer infestation, the Ist instar larvae were released on stem pieces of mature plants. Although the larval survival on all transgenic plants was significantly different among each other but in some transgenic lines (T-TNG-61, T-TNG-26, T-TB-25 and PB-19-J1) it ranged from 31.11 to 48.88 per cent after four days (Table 5). The mortality

percentage was of lower order, compared to that caused by the respective transgenic during vegetative stage. The development of surviving larvae reared on all transgenic plants was very slow and larvae appeared to be unhealthy. This indicated that level of proteinase inhibitor varied with plant growth stage, this observation is in tune with those of Wolfson and Murdock (1990b). It was reported by these workers that younger plant produced higher level of proteinase inhibitor. The systemic accumulation of proteinase inhibitors in non-wounding plant parts diminishes in aging plants and does not occur in mature plants over 30 day old, suggesting the involvement of proteinase inhibitors in plant defense during the restricted period of early development. Daily wounding and insect feeding on mature tobacco and tomato plants did result in a rapid increase in trypsin inhibitor levels in the wounded leaf itself (Jongsma *et al.*, 1994; Alarcon and Malone, 1995). This indicates that proteinase inhibitors can be induced locally by wounding at any time of plant growth stage but the ability to induce proteinase inhibitors systemically in all the leaves is apparently lost as the plant matures. This may be due to physiological constraints in the plant. On the other hand, the inducible defense represents a fine balance between allocation of the limited resources into growth and production and alternatively allocation into an effective defense.

Young plants which are fragile might invest their resources into a systemic defense following wounding but as the plants mature such an investment may not

be desirable unless the infestation level is very high. Alinia *et al.* (2000) reported the decline in Cry 1Ab titer in both leaf sheath and blade at the reproductive stage of the rice plant in comparison with the vegetative stage. The decline in toxin level of line 827 corresponded with the insect resistance decline. This reduction in toxin level may be related to morphological changes during development such as decline in proportion of mesophyll cells in relation to vascular tissues and reduced photosynthesis may also be contributing factor.

Studies on the survival of lepidopterous rice pests on Cry 1 Ab-transformed aromatic rice indicated that these transformed plants were more resistant to young larvae of *S. incertulas*, *Chilo suppressalis* (Walker) and *C. medinalis* than control plants at the vegetative stage but not at the flowering stage of plants (Alinia *et al.*, 2000). Their findings indicated non-significant differences with respect to survival of 10-day old larvae both at vegetative and flowering stage. Alinia *et al.* (2000) reported the retardation of development of *C. suppressalis* larvae on Cry 1Ab transformed aromatic rice plants. Decline in Cry 1Ab levels at reproductive stage of plants was also reported.

5.2.2 Response of transgenic rice plants using whole plant bioassay

The whole plant bioassay for rice leaffolder during vegetative stage of plants

In case of rice leaffolder whole plant bioassay, seven transgenic plant numbers were found resistant with a damage score 3, and adjusted damage rating

ranging from 13.26 to 24.02 per cent (Table 6). On control plants, damage rating was 3 to 4-fold more as compared to all transgenic plants.

While comparing the results of leaf-section bioassay and whole plant bioassay, it was found that two methods gave similar inference, particularly when both larval mortality and leaf area consumed were used as the criteria for assessing resistance in the leaf-section bioassay. This finding derives support from the work of Ye *et al.* (2000) who reported that leaf-section bioassay and whole plant bioassay for stem borer (*C. suppressalis*) resistance in transgenic rice containing a synthetic Cry 1Ab gene from *Bacillus thuringiensis* produced similar results when larval mortality, and leaf area consumed were considered for resistance assessment.

The whole plant bioassay for stem borer during vegetative and earhead stage of plants

During vegetative stage, the plants were analysed after one month of insect infestation by using the standard technique given by IRRI (Anonymous, 1988). The six transgenic lines (T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82 and PB-18-J1) were found resistant with damage score 3 (38.20 to 40.19 damage rating) and the other four were found moderately resistant with damage score 5 (46.93 to 50.07% dead hearts) (Table 7). There may be a difference of proteinase inhibitor protein levels among the transgenic plants which have shown the variable resistance level in plants as also observed by Duan *et al.* (1996). It was observed

that 65 per cent plants of one generation produce similar *pin II* protein levels to their parents and 35 per cent produce twice as much *pin II* protein as their parents.

During earhead stage, the plants were dissected after 14 days of insect infestation. Only three transgenic lines (T-TNG-64, T-TNG-61 and T-TB-82) were found resistant with damage score 3 (Table 8) on the basis of per cent white heads and other seven were found moderately resistant (damage score 5). This might be due to diminishing level of *pin II* during maturity as opined by Wolfson and Murdock (1990b) who reported that plant age has a much greater impact on the ability of the plants to support the growth of *Manduca sexta*.

The number of larvae survived at earhead stage were more but these were poor in growth and looked sickly with reduced weight and dimension (Table 9). Duan *et al.* (1996) also reported retardation in growth and development in terms of weight decrease and development restricted to 2nd-3rd instar stage, contrast to 3 to 4 fold weight increase and development to the 4th or 5th instar stage on control plants. Similar type of results have been reported with respect to transgenic rice plants expressing trypsin inhibitor. When incorporated into an artificial diet, both proteinase inhibitors, soybean trypsin inhibitor and potato proteinase inhibitor II significantly reduced the growth and development of larvae of *Heliothis zea* (Boddie) and *Spodoptera exigua* (Hubbner) (Broadway and Duffey, 1986). However, these proteinase inhibitors had no effect on the *in vivo* digestion of protein indicating that these do not reduce the level of digested protein. It was

also elaborated by these workers that when the larvae chronically ingest the protein inhibitors, there was significant elevation of the tryptic activity. Thus, the pernicious hyperproduction of trypsin coupled with insufficient dietary availability of sulphur containing amino acid (i.e. methionine) needed for enzyme synthesis result in inhibition of the growth.

Mochizuki *et al.* (1999) recorded that the weight of *C. suppressalis* larvae on the transgenic lines expressing WTI-1B at 1 or 2 ng/10 µg was significantly less than the control plant but larval weight on the lines at 0.5 ng WTI-1B/10 µg did not differ with the control. They stated that growth and development of insects were negatively affected by anti-nutritional effect of proteinase inhibitors. Further, a delay in larval growth on the transgenic lines will make the pest more susceptible to natural enemies and pathogens under field conditions.

5.3 Biology of rice leaffolder

Adverse effects of proteinase inhibitor (*pin II*) on various biological parameters of the insect account for antibiosis mechanism of resistance (Wolfson and Murdock, 1995). Various larval and pupal parameters were studied on 10 transgenic plants which showed *pin II* positive expression in present studies. Larval survival was 30.00 to 48.00 per cent on all transgenic as against 84.00 to 92.00 per cent on control plants (Table 10). These findings suggest strong expression of antibiosis particularly in T-TB-82, T-TNG-64, T-TNG-61, T-TNG-46b and T-TB-95 lines where only 30.00 to 36.00 per cent larvae survived.



Besides larval survival, larval development was also influenced by transgenic plants containing potato proteinase inhibitor II gene. The larvae were restricted to 2nd and 3rd instar for a long period and the larval period extended maximum to 29.50 days on T-TB-82, which was at par with T-TNG-46b, T-TNG-61 and T-TNG-64. Wolfson and Murdock (1987) found that proteinase inhibitors if active against proteases of target insect, can delay larval development and not cause direct mortality. While studying the effect of consuming low doses of E-64, a cysteine proteinase inhibitor on life history parameters of a Mexican bean beetle, *Epilachna varivestis* Mulsant, Wolfson and Murdock (1995) observed that E-64 adversely affect biological parameters of the insect, for instance, prolongation of larval development and pre-oviposition period. Larval weight was also reduced due to their poor growth. These observations are in tune with the reports that CpTI expressing plants showed reduced damage compared to control plants and reduced insect survival and biomass (Hoffman *et al.*, 1992). The present findings are in conformity with those of Wolfson and Murdock (1987); Xu *et al.* (1996); Duan *et al.* (1996); Mochizuki *et al.* (1999). Gatehouse *et al.* (1997) have also reported poor larval growth and development when larvae reared on transgenic plants containing proteinase inhibitor gene.

Pupation was low (10.00 to 26.00 per cent) on all transgenic plants, compared with control TNG67 (92.00%), Taraori Basmati (90.00%) and Pusa Basmati (84.00%) plants. Consequently pupal weights were also lower on

transgenic plants and pupal period was extended on these plants (Table 11). Moth emergence was also significantly less on the transgenic rice plants as compared to non-transgenic plants. Results with respect to effect of transgenic on biological parameters indirectly support the findings of Bolter and Green (1997) who observed the adverse effect of E-64 on larval growth, development of pupae and survival of adults of colorado potato beetle. Similar type of effect on biological parameters of Mexican bean beetle, *E. varivestis* feeding low dose of E-64, a cysteine protease inhibitor were observed. The effects were expressed as prolongation of larval and pupal periods of the insect. However, no effect on pupal weight and larval mortality was reported by these workers (Wolfson and Murdock, 1995).

5.4 Mechanisms of resistance

5.4.1 Rice leaffolder larvae feeding on transgenic rice plants

Mean leaf area damage in the test plants by 4th instar larvae ranged from 235.60 mm² in T-TB-82 transgenic lines of Taraori Basmati to 435.20 mm² in TNG67 (Table 12). There was a significant difference between transgenic plants and control plants with respect to extent of leaf area consumed. However, Wolfson and Murdock (1995) have reported a non-significant difference in larval leaf consumption for control and inhibitor-fed insect on E-64, expressing cysteine protease inhibitor.

5.4.2 Rice leaffolder and stem borer oviposition on transgenic plants

There was no difference in number of eggs and egg masses laid by adult of both insects on transformed and non-transformed plants (Table 13 and 14). No change was recorded in morphological characteristics of plants due to transformation of gene (*pin II*) in plants.

Therefore, it may be concluded from the present studies that five transgenic plant no. viz., T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95 and T-TB-82 were found resistant to rice stem borer and rice leaffolder because of antimetabolic effects of proteinase inhibitors on insects digestive system. Larval feeding, and larval and pupal parameters were affected adversely on these transgenic plants carrying potato proteinase inhibitor II gene. Review of work on protease inhibitors indicate that these compounds, if active against the digestive proteases of the target insect, can delay larval development (Wolfson and Murdock, 1987). The antibiotic activity of protease inhibitors is assumed to derive from inhibitor interference with protein digestion, which decreases amino acid availability. Low availability of amino acids could then directly depress the synthesis of protein necessary for growth, development or reproduction. Alternatively, the inhibitors could affect development indirectly by way of a feedback mechanism that initiates increase production of digestive proteases to compensate for the low levels of available amino acids (Broadway and Duffey, 1986).

The prolongation of the development period and reduction in adult emergence of leaffolder when fed on transgenic rice plants as observed during the present investigation, are of much significance. This was earlier suggested by Gould (1986) that the depressed population growth rates caused by transgenic plants would be most effective against multivoltine insects, which usually reach economic threshold during the second (or later) generation in a season. Boulter (1993) reported advantage by delaying the development of resistance to the protease inhibitors within the insect population as well as retaining the anti-insect effect for a much longer period.

The expression of the induced proteinase inhibitor in tomato leaves depends on the environmental conditions to which the plants are exposed at the time of wounding or insect feeding (Wolfson and Murdock, 1990b). There were some evidences of reduced expression of proteinase inhibitors. These might be due to insect adaptation to the ingestion of proteinase inhibitors and expression of an anti-sense gene in transgenic crop plants which reduced the resistance level of transgenic plants. Larocque and Houseman (1990) reported that different effects of varying inhibitors and differences in the biochemical interaction between inhibitor and insect proteases are important considerations for understanding the potential antimetabolite effects.

CHAPTER – VI

SUMMARY AND CONCLUSION

In the present studies, ten transgenic rice lines *viz.*, T-TNG-64, T-TNG-61, T-TNG-46b, T-TNG-26, T-TB-95, T-TB-82, T-TB-57, T-TB-25, PB-18-J1 and PB-19-J1 containing *pin II* gene (potato proteinase inhibitor II) were evaluated for their response against rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and rice stem borer, *Scirpophaga incertulas* (Walker), during the year 2001 and 2002. These transgenic lines of rice were produced by *Agrobacterium* gene transformed method in Japonica cultivar, Taraori Basmati and Pusa Basmati by the Department of Biotechnology and Molecular Biology, CCS HAU, Hisar. RKJ1 plasmid containing the *pin II* gene was used for genetic transformation. First of all the transgenic nature of plants of T₁ and T₂ generations during the paddy season of 2001 and 2002 was analysed by using Polymerase Chain Reaction (PCR) analysis. The presence of two bands, one for rice actin 1 fragment (304 bp) and another for *pin II* fragment (490 bp) confirmed the transgenic nature of plants. The average segregation ratio between transgenic and non-transgenic was 1:1 in T₁ generation and 2:1 in T₂ generation.

The *pin II* positive transgenic rice lines of T₂ generation were used for further studies i.e. for insect bioassay against rice leaffolder, *C. medinalis* and stem borer, *S. incertulas*. The experiments were conducted in greenhouse approved for growth of the transgenic rice plants and in air-conditioned room. Bioevaluation by different methods such as cut-leaf, cut-stem, whole plant bioassay against the two test insects; mechanisms of resistance in transgenic lines and biology of *C. medinalis* to observe the effect of proteinase inhibitor (*pin II*) gene on the growth and development of the insects were studied in the laboratory and greenhouse.

The cut-leaf bioassay was conducted for both *S. incertulas* and *C. medinalis*. In this method, two small pieces of leaf of growing plants from each of transgenic lines and control plants were kept in petridishes. Five neonate larvae of *C. medinalis* and *S. incertulas* on each leaf section were transferred to petridishes separately for four days. The per cent larval mortality was recorded on second and fourth day and total leaf area fed in mm² recorded after four days. The highest larval mortality of *C. medinalis* (95.00%) was reported on T-TNG-46b followed by T-TB-82 (92.50%), T-TNG-64 (87.50%) and T-TNG-61 (87.50%). The leaf area eaten after four days was lowest on T-TNG-46b and T-TNG-61 (4.12 mm² and 4.50 mm²) as against 18.62, 19.12 and 23.00 mm² in control plants (Pusa Basmati, TNG67 and Taraori Basmati).

Regarding response to *S. incertulas* as assayed by cut-leaf bioassay, the highest larval mortality (95.00%) was observed in T-TNG-61 followed by T-TNG-26 (92.50%), T-TB-82 (92.50%) and T-TB-57 (92.50%) after four days. Likewise, the leaf area eaten was also less on all transgenic rice lines with minimum leaf consumed (9.37 mm²) in case of T-TNG-61. In general, larval mortality on transgenic lines was higher (>85.00%) and leaf area consumed was of lower order (25.25 mm²) in contrast to control plants (4% maximum larval mortality and 35.12 mm², the maximum leaf area consumed).

The cut-stem bioassay was conducted only for rice-stem borer, because stem borer feeds inside the panicle stalk. This experiment was conducted during the earhead stage of plants to observe the effect of foreign gene during the maturity of the plants. The experimental design and method used were same as in case of cut-leaf bioassay. The survival of *S. incertulas* neonate larvae (20 to 49%) after four days was lower on transgenic plants, compared to control plants. However, the larval mortality recorded at this stage was less as compared to that recorded during the vegetative stage of these transgenic rice-lines.

Whole plant bioassay was conducted for *C. medinalis* at vegetative stage while for *S. incertulas* at two stages i.e. vegetative and earhead stage. In these experiments, ten neonate larvae of *C. medinalis* and *S. incertulas* were used for each replication of transgenic line separately. The potted plants were arranged in Complete Randomized Design in aluminium tray (180 cm x 75 cm x 15 cm) filled

with water. There were six replications for each insect. Each pot or replication was covered individually with a cage (85 cm x 36 cm diameter) and the Standard Evaluation System for rice was used for the damage rating and damage grade. In case of *C. medinalis* during vegetative stage of plants, the observations were recorded after 16 days of releasing the neonate larvae. The damage rating was recorded on the basis of leaf damage. Seven transgenic rice lines viz., T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82, T-TB-57 and T-TB-25 were found with a damage grade of 3 with 15.79 to 24.02 per cent adjusted damage rating and other three secured damage grade of 5 with adjusted damage rating 33.55 to 39.62 per cent, compared to 9 damage grade and 100 per cent damage rating in control plants.

For whole plant bioassay for *S. incertulas* during vegetative stage of plants, the observations were recorded after one month. Six transgenic lines (T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-82, T-TB-95 and PB-18-J1) were found resistant with a damage score of 3 and four transgenic lines (T-TNG-26, T-TB-57, T-TB-25 and PB-19-J1) were found moderately resistant with a damage score of 5. The respective values for damage rating were 38.72, 40.41, 38.56, 39.02, 38.30 and 40.19 per cent for transgenic and 100 per cent for control (non-transgenic plants). As regards the effect of *pin II* gene observed at earhead stage of the transgenic lines on *S. incertulas*, only three transgenic lines (T-TNG-64, T-TNG-61 and T-TB-82) performed better with a damage grade 3 and damage rating ranged from

23.49 to 40.17 per cent. Other seven transgenic lines scored grade 5 and damage rating 42.13 to 54.38 per cent.

Effect of *pin II* gene at earhead stage on survival of *S. incertulas* larvae was determined. For this, the plants were dissected after 14 days. The per cent survival on transgenic lines was higher as compared to vegetative stage of plants but significantly different from control plants. The larval survival on the transgenic lines was recorded in the range of 30.00 to 44.00 per cent. The lowest larval survival (30.00%) was recorded on T-TB-82 which was at par with T-TNG-64 (36.00%) and T-TB-95 (36.00%) whereas maximum (92.00%) on control plants, Taraori Basmati and Pusa Basmati. Likewise, surviving larvae gained less weight (9.00 to 10.40 mg) and dimension (2.50 to 4.90 mm²) on these transgenic rice lines as compared to weight gain (27.60 to 34.60 mg) and dimension (27.00 to 36.40 mm²) of larvae on TNG67, Taraori Basmati and Pusa Basmati. In addition, development of the surviving larvae was restricted to 2nd to 3rd instar as compared to 4th to 5th instar on control plants.

Biology of *C. medinalis* on transgenic lines was studied as per method described for whole plant bioassay conducted for *C. medinalis*. It was recorded that larval survival, larval period, larval weight and pupal period, weight and pupation were influenced to varying extent by transgenic rice lines. Lowest larval survival (30.00%) was recorded on T-TB-82 which was at par with T-TNG-64, T-TNG-61, T-TNG-46b and T-TB-95 whereas highest (92.00%) was recorded on

control plant, TNG67. Longest larval period and less larval weight were recorded on the tested transgenic lines which differed significantly from that on control plants. Per cent pupation of the surviving larvae was less on all the transgenic plants than control plants. Lowest pupation was recorded on T-TNG-46b (10.00%) followed by T-TB-82 (12.00%) and highest (92.00%) on TNG67. Pupal period was highest (16.80 days) on T-TB-25 followed by T-TNG-46b and T-TB-82 (15.80 days). Similar type of results were obtained with respect to pupal weight. Moth emergence was significantly low on the transgenic plants than control plants.

To study the mechanism of resistance in transgenic lines, effect on larval feeding and adult oviposition of *C. medinalis* and *S. incertulas* was studied. In case of larval feeding, one 4th instar larva of *C. medinalis* was released on the potted plants and each pot was covered individually with a nylon cage. Larval feeding after 48 h was significantly less on transgenic lines as compared to control plants. Lowest larval feeding (235.60 mm²) was recorded on T-TB-82.

Adult oviposition was studied for both insects. In this, five pairs of adult were released on each pot for each insect separately and each pot was individually covered with a nylon cage. After 48 h, the eggs and egg masses of leaffolder and stem borer were counted. There was no distinct difference in oviposition on the transgenic line and non-transgenic.

It may be concluded from the studies that there was significant effect of transgenic rice lines i.e. proteinase inhibitor (*pin II*) on growth and development of *C. medinalis* and *S. incertulas*. The extent of damage by both the test insects was less on these transgenic lines. The five transgenic lines viz., T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95 and T-TB-82 were found most resistant to both rice leaffolder *C. medinalis* and rice stem borer, *S. incertulas*.

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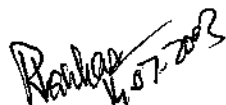
ABSTRACT

1. **Title of thesis** : Response of transgenic rice plants to rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and stem borer, *Scirpophaga incertulas* (Walker)
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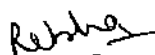
(An abstract of the dissertation submitted to the CCS Haryana Agricultural University, Hisar in partial fulfillment of the requirements for the degree of Ph.D.)

Ten transgenic rice lines (*Oryza sativa* L.) transformed with *pin II* (potato proteinase inhibitor II) gene namely, T-TNG-64, T-TNG-61, T-TNG-46b, T-TNG-26, T-TB-95, T-TB-82, T-TB-57, T-TB-25, PB-18-J1 and PB-19-J1 were evaluated for their response against rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) and rice stem borer, *Scirpophaga incertulas* (Walker) during 2001 and 2002 in rice season under greenhouse and laboratory conditions. Firstly, the transgenic nature of plants of T₁ and T₂ generations (a plant derived from tissue culture of transformed embryogenic callus is referred to as a T₀ plants, thus T₁ and T₂ plants are 2nd and 3rd generation after transformation) were analysed by Polymerase Chain Reaction (PCR) analysis. The presence of two bands, one for

rice actin 1 fragment and another for *pin II* fragment confirmed that the lines under test were transgenic positive. The ratio between transgenic and non transgenic plants and stability of *pin II* gene were improved in the T₂ generation, as shown by molecular analysis. The *pin II* positive plants of T₂ generation were used for insect bioassay during vegetative and earhead stage of plants. For this, the various methods viz., cut-leaf, cut-stem and whole plant bioassay were employed. Biological parameters of leafhopper on transgenic lines and mechanism of resistance in the transgenics were studied. Firstly, the short period methods like cut-leaf and cut-stem bioassays were conducted in the petridishes against neonate larvae. With respect to response of both insects, highest larval mortality and lowest leaf area damage was recorded on T-TNG-61, T-TNG-46b, T-TNG-64, T-TB-82, T-TB-95 and T-TB-57 during the vegetative stage of plants. In cut-stem bioassay for *S. incertulas* during earhead stage of plants, the larval survival was more as compared to that at vegetative stage of the transgenic plants but with poor development of larvae. With regard to whole plant bioassay, in case of *C. medinalis* at vegetative stage of plants, the plants were evaluated for resistance after 16 days. Seven transgenic lines viz., T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82, T-TB-57 and T-TB-25 were found resistant with 3 damage score and adjusted damage rating within 13.26 to 24.02 per cent. In case of *S. incertulas*, the observations were recorded after one month and six transgenic lines (T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95, T-TB-82 and PB-18-J1) were found resistant with a damage score of 3 (38.30 to 40.41% adjusted damage rating). Others were found moderately resistant with 5 damage score (57.69 to 59.19% adjusted damage rating). The whole plant bioassay during earhead stage showed that there may be dilution of effect of the gene at maturity of the plants. Per cent larval survival at this stage increased in comparison with vegetative stage but larval weight and dimensions were reduced. Antibiosis mechanism of resistance in the five transgenic rice lines (T-TNG-64, T-TNG-61, T-TNG-46b, T-TB-95 and T-TB-82) was found for both insects. The extent of damage by both the test insects was less on these transgenic lines.

 07-2003

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