

**Reverse and Forward Genetic Approaches for the Development of Disease
Resistant Wheat (*Triticum aestivum* L.).**

Dissertation

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DEDICATION

To my honourable parents and grand parents

SUMMARY

Wheat (*Triticum aestivum* L.) is one of the most important crops of the world and ranks first in the area covered worldwide and second after maize in production. It is prone to many diseases the most important of which are fungi. For example, fusarium head blight (FHB) and powdery mildew (PM) of wheat are the destructive diseases of wheat especially in the cool and humid areas of the world. Classical wheat breeding programmes have produced some cultivars that resist against FHB and PM to some extent. But due to the ever mutating pathogens and vertical nature of the resistance these cultivars also become susceptible after a few years. The development of the genetic engineering has given a passage to the scientists and wheat breeders to look for the ways that can help them to develop a genotype which can stably resist against more than one pathogen with non race specificity. For this purpose it was decided i) to co-express two antifungal genes (*HarChit* and *HarCho*) under constitutive *Ubi* and stress/disease inducible *Vst1* promoter and ii) knock down three members (*Ta-GSL3*, *Ta-GSL8* and *Ta-GSL10*) of Glucan Synthase Like gene family in order to find out their role in disease resistance.

A total of 9 lines were developed with co-integration and expression of *HarChit* and *HarCho* under constitutive *Ubi* promoter (4 lines) and inducible *Vst* promoter (5 lines). The integration pattern showed single copy as well as multi-copy integration of both the genes. The copy number varied for *HarChit* (1-3) and *HarCho* (2-10). Inducible promoter seemed to have no affect on transformation. Pathological testing showed a decrease in the susceptibility for both the pathogens tested. For *Erysiphe Graminis* (*E. graminis*) a decrease in susceptibility was seen upto 75% while for *Fusarium. graminearum* (*F. graminearum*) the decrease in susceptibility was seen upto 58%. All the primary transformants with the exception of a couple showed normal growth.

Only 4 (No plant for *Ta-GSL10*) transgenic lines were found for the knock down of *GSL* genes and that too when siRNA forming DNA fragments of around 150 bp were used in the RNAi constructs. No transgenic plant was found when larger siRNA forming DNA fragments were used in the RNAi constructs. Out of 4 plants only two (1 for *GSL3*, 1 for *GSL8*) showed a reduction in gene expression in T₀ and T₁ generations. Pathological analysis with *F. graminearum* showed an increase in disease susceptibility of up to 60% for *Ta-GSL3* knock down and 40% for *Ta-GSL8* knock down. This shows the involvement of both of these genes in disease resistance.

Due to the difficulty in getting knock down lines using RNAi cassettes under constitutive promoter, it was decided to find out disease inducible genes in wheat. Four genes inducible

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under disease infection were identified in wheat under *F. graminearum* infection; the corresponding promoter of those can be identified and used in future transformation experiments.

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ABBREVIATIONS

ABBREVIATIONS

°C	degree Celsius
2,4-D	2,4-Dichlorophenoxyessigsäure
<i>A. tumefaciens</i>	<i>Agro-bacterium tumefaciens</i>
aa	amino acids
Avr-Gen	Avirulence gene
BC	Before Christ
bp	base pair
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CIM	Callus Induction Media
cm	centimeter
CSPD	3-(4-methoxyspiro{1,2-dioxetan-3,2'-(5'chloro) tricyclo [3.3.1.13,7]decan}-4-yl) Phenylphosphate, Disodium salt
dag	days after germination
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxyribonucleoside 5'-triphosphates
DON	De-oxynivalenol
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. graminis</i>	<i>Erysiphe graminis</i> f.sp. <i>tritici</i>
EST	expressed sequence tag
EtBr	Ethidium bromide

ABBREVIATIONS

<i>F. graminearum</i>	<i>Fusarium graminearum</i> L.
FAO	Food and Agriculture Organization of the United Nations
FC	Fertile Crescent
FHB	Fusarium Head Blight
g	gram
GUS	b-Glucuronidase
h	hour
He	Helium
HR	Hypersensitive response
IG	Digoxigenin
kb	kilo basepair
kDa	kilo dalton
m	meter
M	molar
Min	minute
mJ	millijoule
ml	millilitre
mm	millimeter
mM	millimolar
mmol	millimole
mRNA	messenger RNA
mRNA	messenger RNA
ng	nanogram
Niv	Nivalinol
nm	nanometer
nM	nanomolar
Nos	nopaline synthase
p.A.	per analyse

ABBREVIATIONS

Pa	Pascal
PAT	Phosphinothricin acetyl transferase
<i>pat</i> -gene	Phosphinothricin-acetyltransferase gene
PCR	polymerase chain reaction
Pfu	plaque forming unit(s)
pg	picogram
PM	Powdery Mildew
Poly(A+)	mRNA polyadenylated mRNA
PR-Genes	Pathogenesis related genes
PR-Proteins	Pathogenesis related proteins
psi	pound per square inch
<i>R</i> -Gen	Resistance gene
RIP	Ribosome inactivating proteins
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	Reactive oxygen species
rpm	rounds per minute
RT	reverse transcription
SA	Salicylic acid
SAR	Systemic acquired resistance
sec	second
SM-I	Selection media-I
SSC	sodiumchloride-sodiumcitrate
T ₀	Direct transgenes regenerant
T ₁	First generation from to self crossing
T ₂	Second generation from T ₁ self crossing
TBE	Tris/Borate/EDTA electrophoresis buffer
temp.	temperature
T _m	melting temperature

ABBREVIATIONS

U	unit (restriction enzyme)
<i>ubi1</i> -Promotor	Ubiquitin1-Promotor
<i>uidA</i> , <i>gus</i> -gene	β -Glukuronidase gene
Wt	Wild type
X-Gluc	5-Bromo-4-chloro-3-indolyl- β -D-glukuronsäure
μ g	microgram
μ l	microliter
μ m	micrometer
μ M	micromolar
UV	ultraviolet light

INTRODUCTION

1. INTRODUCTION:

1.1 Wheat and the World Food Security:

Wheat is one of the most important food crops in the world. It is grown through out the world and ranks first among all agricultural crops with the covered area of 2.81 million hectares in 2007. Regarding production it ranks second among cereals after maize with the world production of 611 million metric tons (FAO, 2008). It is an essential source of calories and proteins in human diet. It also provides vitamins *i.e.*, B and E, Magnesium, Phosphorus and fibre as well.

Healthy and nutritive food is the basic right of every individual and the availability of this food to every human being is called world food security. World food security is a far more complex phenomenon than it was a few generations ago. The world population has been increasing ever since and doubled in the last 50 years. The world population is over 6.5 billion and is projected to reach 9 billions by the year 2050. Every year more than 76 million are being added to this number. Nine out of every ten children are born in developing countries (Annonymous, 2008). Increase in population means increased food needs. In the former times increased food needs had been fulfilled by simply increasing the land area under cultivation and little attention was given to increase the yield. Under present circumstances no country in the world has a scope of increasing agricultural land area except Brazil but that will be the cost of many biological species found in its forests. In the recent past some countries have been using their reserve stocks to fulfil their needs. China, once having a big stock of grains, has become the biggest importer of wheat and other grains over night.

Food security is in danger, primarily due to desertification, housing and urbanisation of agricultural land, climate change, water shortage and bio-fuel production. Decrease in the cultivated area can be stopped by proper planning of the towns and cities as well as by customising the housing plazas, where a large number of people can live on a small piece of land. This should specially be promoted in developing countries. Climate change is postulated to be the outcome of industrialization, specially the burning of carbon fuel that has resulted in high level of atmospheric CO₂, which resulted in the shrinkage of fisheries, erosion of soil, increase in average annual temperature, un-ordered annual precipitation, melting of glaciers, rising level of seas and dryness of rivers. The ultimate effect is adverse changes in plant macro and micro environment. Plant ecologists have analysed that an increase of 1°C results in 4 percent decrease in the production of wheat (Brown, 2005). The issue of water shortage is

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in fact more severe than the issue of reduction in carbon fuel. Man can not survive with out water which is evident from the fact that an individual drinks 4 quarts of water in a day but the food he eats is produced by using 2000 quarts. At the time when people in the third world are facing the problems of food shortage and mal nutrition, some economies are trying to use food crops as an alternate to the carbon fuel. This will make the availability of food more difficult to the poor.

Under the present circumstances, it looks like there are few solutions to insure the food security of the world. The scientists working on the climate change worldwide will have to start looking for the solutions to stop abrupt climatic changes rather than just predicting the changes. The other solution lies in the development of crops that can survive in this changing environment. Governments should appreciate people to prefer diets which can be cultivated successfully under the shortage of water and adverse conditions. Wheat, maize and rice are the three leading staple and fibrous food crops in the world and they fulfil 43 percent of the caloric requirements of the world population and have many indirect influences to human life. If we make a comparison among three of them, we will come to know that rice being the most water loving crop will not be fit under water shortage conditions. Maize due to its high cost of production does not suit for the poor farmers of developing countries. Wheat on the other hand is grown through out the world and fits almost in all climatic conditions of Africa, Asia, Europe and America and needs comparatively less water than maize and rice.

Wheat is consumed though out the world as an important part of the diet, *i.e.*, in the form of bread, noodles, spaghetti, cakes, biscuits and other sweets in Europe and America, while in the form of chapatti in Asia and Africa. In Indo-Pak subcontinent wheat is the most popular staple food and is the symbol of food security. Governments are highly disturbed if there is any kind of shortage in the provision of wheat to masses. Political parties include improvement in wheat production and provisions in their manifestoes. In addition to this, it is also an important commodity of smuggling among India, Pakistan and Afghanistan. From these facts it can be estimated that any step towards the betterment in the production of wheat means a step towards world food security.

1.2 Origin And Evolution Of Wheat With The Challenge Of Diseases:

The exact origin of the present day wheat is not known. Fossil records date wheat like kernels back to 15000 BC (Briggle, 1981). Wheat originated from wild grasses some where perhaps in the Middle East in an area known as Fertile Crescent (FC) in early historical times. Evidence from the current distribution of cereal land races point out the “hilly flanks”

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surrounding the deserts and steppes of Syria, Iraq and Iran as the area, in which domestication of wheat started. Archaeobotanical work points not only the presence of domesticated crops in the villages of FC in 7500 BC but also the spread of these crops into South Eastern Europe (Nesbitt and Samuel, 1996 and Hansen, 1992). Recently, Isoenzyme studies and RFLP analysis showed that present day European bread wheat is not simply the domestication of migrated wheat from Asia but a separate process of evolution was also going on in the Europe (Dvorak *et al.*, 1999).

Einkorn was perhaps the first diploid cultivated wheat specie. Its cultivation was abandoned in the Bronze Age and people started to cultivate tetraploid species which were easy to harvest and favourable to cultivate in warm climate. Tetraploid species existing today are believed to be the progenitors of *T. dicoccoids* (with AABB genome) which later on accidentally crossed with *Aegilops tauschii* (with DD genome) to give rise to present day hexaploid wheat, (with AABBDD genome) *Triticum aestivum* (Salamini *et al.*, 2002).

Wheat is attacked by many pathogens including fungi, bacteria and viruses at almost all stages of growth and development. Fungal diseases have been serious threat perhaps since the start of the domestication of wheat. Wheat rusts, smuts, powdery mildews and scabs are among the main fungal diseases found almost through out the world in wheat growing areas. These diseases cause from little damage to 70 percent of crop loss in terms of grain weight but quality is also deteriorated by fungal secreted toxins (Naz, 2007). Wheat land races especially with durum origin have resistance genes but unfortunately when these genes are bred to commercial bread wheat they do not perform as expected. This may be due to the presence of some suppressors in bread wheat which do not let the genes to create resistance against diseases (Kerber and Aung, 1995; Knott, 2000; Knott *et al.* 2005; Bonman *et al.* 2007).

In most of the wheat breeding experiments disease resistance has been the major breeding objective. It was the objective to bring disease (rust) resistance in wheat, when in the middle of 20th century Dr. Norman E. Borlaug bred semi-dwarf wheat which boosted the yield up to six times of the existing. This work was termed as “Green Revolution” and he was awarded the Nobel Prize of peace in 1970. But interestingly, disease resistance issue is still there and in the era of plant transformation it gives a chance to plant scientists for evaluating innate disease mechanisms and try some mechanisms in wheat from across the species for improved disease resistance. In this work, the focus will be built on two diseases caused by pathogens with biotrophic and necrotrophic mode of infection.

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1.2.1 Fusarium Head Blight (FHB) Of Wheat:

FHB was described in England at the end of nineteenth century and was considered as a major threat to wheat and barley production during the early twentieth century. Recent outbreaks in Asia, Canada, Europe and South America have helped to identify FHB as a major limiting factor to wheat production in many parts of the world. FHB has approximately caused an estimated economic loss of 2.7 billion dollars in Midwestern United states during 1998 and 2000. (Dubin *et al.*, 1997; Stack, 1999, 2003; Muriuki, 2001; Nganje *et al.*, 2004)

FHB causes direct yield losses because of the sterility of florets and shrivelled, light test-weight kernels (Figure. 1). Furthermore, infected grain is often contaminated by trichothecenes and estrogenic mycotoxins also known as deoxynivalenol (DON) and nivalenol (NIV) (McMullen *et al.*, 1997; Qu *et al.*, 2008) that are hazardous to animals and humans and provoke indirect health damages (Marasas *et al.*, 1984). Many countries of the world have already started to legislate on the amount of mycotoxins present in the grain. In the USA, an economically important part of the crop is rejected by the industry because of DON (estrogenic mycotoxin) contamination.

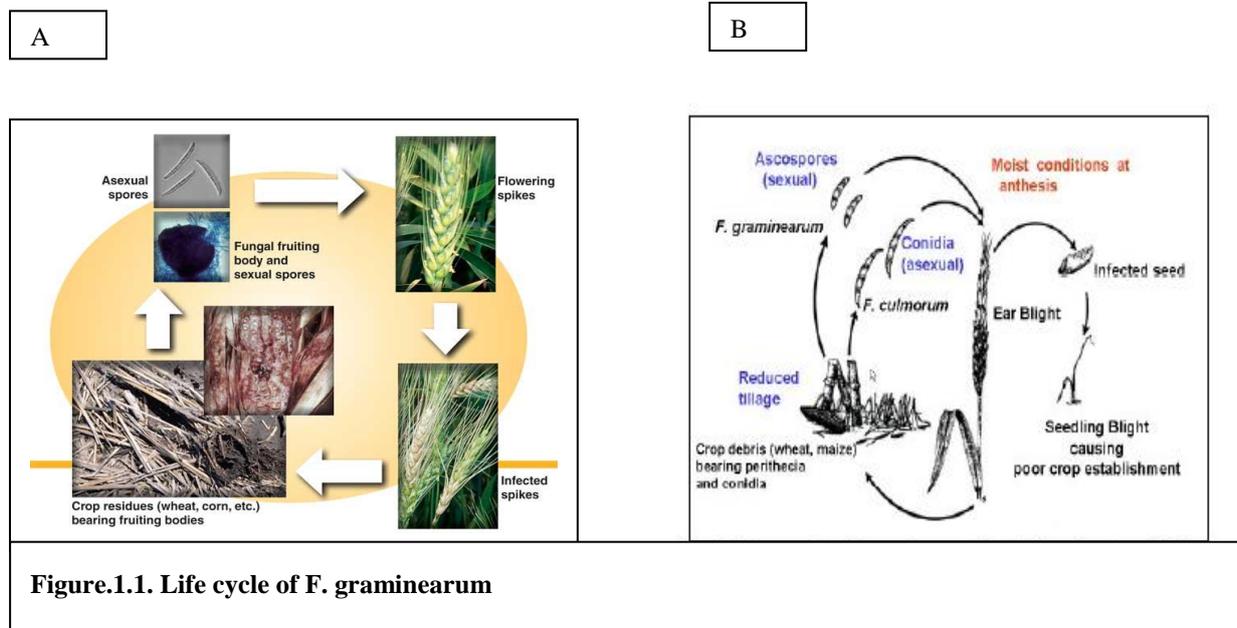
FHB of wheat is caused by several species of the genus *Fusarium*. Most common among these causal organisms is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch). *Fusarium graminearum* (*F. graminearum*) is a necrotrophic, filamentous ascomycete. *F. graminearum* is a broad host range fungus that can infect wheat as well as many other cereal crop plants, especially barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) (Desjardins *et al.*, 1996; McMullen *et al.*, 1997 and Voigt *et al.*, 2005). FHB is recognised on immature heads where some spikelets or the whole spike looks prematurely bleached. If the rachis is infected, the whole tissue above that point is faded. Mycelium and spore masses can be seen at the base of the diseased spikelets.

The *Fusarium* pathogens have no specialized structures to penetrate into the host cell, like appressoria or haustoria. Instead, the fungus either enters the host through natural openings (Pritsch *et al.*, 2000) or penetrates the epidermal cells directly with short infection-hyphae (Wanyoike *et al.*, 2002). Anthers are reported as the primary infection site where spores of fungus may land and then grow into the kernels, glumes, or other head parts. Once inside the tissue, *F. graminearum* is able to spread systemically. It has been postulated that *F. graminearum* infects its hosts with the help of secreted cell wall degrading enzymes (CWDE). Sub-cuticular growth of the fungus continues and finally, the tissue is abundantly colonized intra and intercellularly (Kang and Buchenauer, 2000a and 2000b; Pritsch *et al.*, 2000;

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Wanyoike *et al.*, 2002). In order to establish their position on the plants *Fusarium* fungi live epiphytically without causing any disease symptoms (Clement and Parry, 1998). When the plant becomes weak, they have a competitive advantage and are already established.

Inoculum for FHB may result from a number of different sources, including crop debris, *F. graminearum* infections on other parts of the plant, infected seed, and may spread from adjacent fields (Carter *et al.*, 2000). Initial infection starts from the primary inoculum which over winters on host residues *i.e.* grass residue, cornstalks and wheat stubbles. Conidia or ascospores are carried from these sources by air currents to wheat heads. During moist and warm weather the spores germinate and invade flower parts, glumes or other parts of spike. Infection is frequent and serious at anthesis. The blight symptoms develop within three days after infection when the temperatures range between 25 and 30 °C at continuous high level of humidity (Brennan *et al.*, 2005).



There are some resistance sources found, especially Chinese and Brazilian. Chinese sources are genetically different from Brazilian sources (Singh *et al.*, 1995; Van Ginkel *et al.*, 1996). These sources are being used in breeding programmes but still there is no genotype completely resistant against FHB. Identification of new sources from within the plant and across species means reduction in disease and improvement of grain quality and quantity.

1.2.2 Powdery Mildew Of Wheat:

Powdery mildew (PM) of wheat is known as a serious disease of wheat as well as other grasses for centuries. It is caused by *Erysiphe graminis* f.sp. *tritici*, an ascomycete that

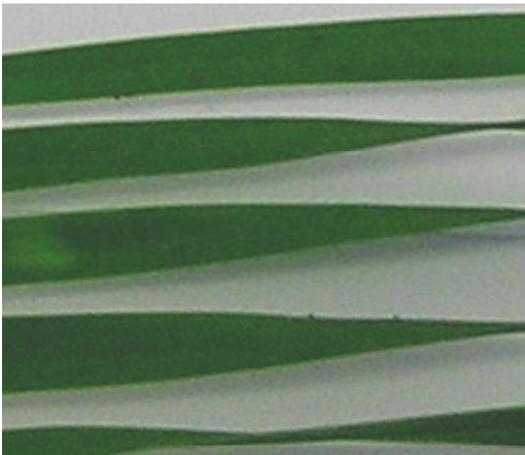
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produces barrel shaped conidia in chains. The sexual stage consists of round, brown fruiting bodies called “cleistothecia” which contain 15-20 asci. The Fungus over-winters in the form of cleistothecia. *Erysiphe graminis* (*E. graminis*) is the causal organism in almost all the grasses but the strain is different for each species and is termed as form species “f.sp”.

E. graminis forms patches of white to grey powdery coating that consists of mycelium and conidia on all the above ground parts of the plant *i.e.* leaves, stem and heads. As the season progresses dark cleistothecia develop in the mycelium (Fig. 1.2B)

The chlorotic patches develop on the diseased leaves but the area below the fungal masses remains green and healthy. This pattern of symptom development is called “Green Iceland” effect. Due to high humidity around them the lower leaves are severely affected as compared to the upper parts (Purdy, 1967; Kingsland, 1982).

A. Freshly inoculated with *E. graminis* f. sp *tritici*



B. 21dpi with *E. graminis* f. sp *tritici*

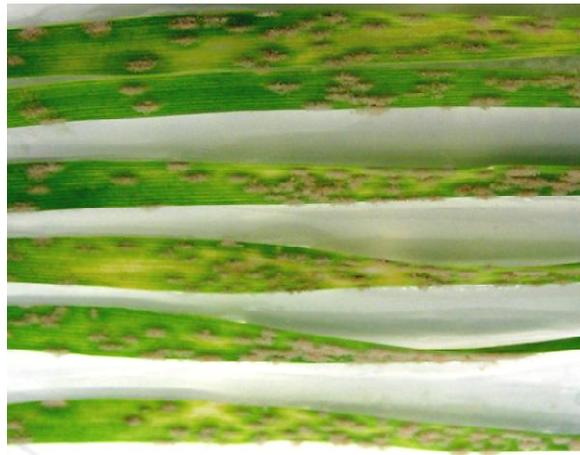


Figure. 1.2. Symptoms of Powdery Mildew

Wind or airborne conidia/ascospores are the primary source of inoculation. The fungus requires a relative humidity of 85-100% and a temperature of 16-20 °C for spore germination. A temperature of more than 25°C affects fungal growth. The fungus penetrates into the epidermal cells through germ tube and forms a specialised structure called haustoria for food absorption. Depending upon severity and attack, PM not only reduces the photosynthetic area and activity but also affects nitrogen re-translocation from the leaves. Root development and leaf assimilation per unit area are also affected. It means all the three components of the yield are negatively affected resulting in reduced yield. (Fried *et al.*, 1976; Carver and Griffiths, 1981; Gair *et al.*, 1983; Kišpatic, 1984; Bowen *et al.*, 1991; Henry and Kettlewell, 1996; Cvjetković, 2003; Samobor *et al.*, 2005, 2006; Lipps *et al.*, 1986; Shi *et al.*, 1998)

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Improved agronomic tools in wheat production *i.e.*, higher population density and higher nitrogen application, create favourable conditions for pathogen development and higher crop sensitivity to diseases (Samobor *et al.*, 2006). More than thirty two powdery mildew resistance loci (*pm-1* to *pm-32*) with forty eight genes / allele have been identified in wheat and many of them belong to wild relatives of wheat (Huang *et al.*, 2000; Liu *et al.*, 2002; Zeller *et al.*, 2002; Xie *et al.*, 2003; Keller *et al.*, 1999). Plant transformation provides an extra edge in evaluating the PM resistance genes from across species and even phyla.

1.3 History Of Wheat Transformation:

Transformation is a tool that can be used to study many aspects of gene function. It is widely used as a routine tool in model crops such as arabidopsis, tobacco and rice. Due to bottlenecks in DNA delivery and tissue culture it is still not a routine technique in wheat. During the last decade, rigorous work has been conducted on Biolistic and *A. tumefaciens* gene delivery systems. Non specificity of the tissue culture has made it possible to transform this recalcitrant crop to some extent (Jones, 2005). The comparative advantage of *A. tumefaciens* mediated transformation is the production of low copy number transgenes. While, using Biolistic bombardment we get normally high copy number integration. This gives rise to gene silencing as well as undesirable rearrangements in the genome (Kohli *et al.*, 2003; Pawlowsky and Sommers 1998 and 1996; and Jones, 2005). Wheat transformation started with transient expression of *gus* gene in cell suspension cultures by Wang *et al.*, 1988 through Biolistic bombardment. Efforts continued by changing and improving different parameters and the first transgenic plant was also reported by Vasil *et al.*, 1992 and Becker *et al.*, 1994. Wheat transformation for the improvement of agronomic traits also started soon (Takumi and Shamida, 1996 and Leckband and Loerz, 1998). In the mean time wheat transformation via *A. tumefaciens* mediation was also started. Cheng *et al.*, 1997 introduced binary vector into wheat using disarmed C-58 strain of *A. tumefaciens* and studied various factors influencing the process. Although the work of Cheng *et al.*, 1997 gave an impetus to the research workers for the establishment of reproducible protocols but even after a decade the routine use of *A. tumefaciens* as transformation agent has not been established. The primary reason is that wheat is not a natural host of *A. tumefaciens* secondly there are some highly specific physical and environmental conditions which are necessary to be provided to wheat explant for getting successful infection and gene delivery by *A. tumefaciens* (Sharawat and Loerz, 2006; Becker, personal discussions). In parallel, transformation by biolistic bombardment is being reported more frequently. There are many reports of successful transformation for over expression and

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gene silencing e.g. Block *et al.*, 1997 created male sterility, Leckband and Loerz, 1998 created disease resistance, Sivamani *et al.*, 2000 created drought tolerance; Oldach *et al.*, 2001; Anand *et al.*, 2003; Mackintosh *et al.*, 2006 and 2007; and Shin *et al.*, 2008 transformed wheat to create disease resistance. Becker *et al.*, 2007 knock downed the expression of alpha Gliadin. Many more transformation projects are going on in the world for the unravelling of basic and applied phenomenon in wheat.

1.4 RNAi As A Tool For Gene Function Analysis / Knock Down Technology In Wheat:

One of the major challenges of plant biology in the post genome era is to determine the exact role of genes in the plant systems. The simplest solution to this problem is to knock down the expression of the gene under study and conclude from the resulting phenotype the role of the gene. Insertional mutagenesis based on transposones and T-DNA insertions (Page and Grossniklaus, 2002, Travella *et al.*, 2006) have been used for this kind of studies. However this approach is applicable only to a few plant species due to the time required to saturate a genome and lethal knock downs. In addition: genetic redundancy, multigene families and polyploidy decrease the efficiency of this approach.

RNAi has been proved as a very efficient tool/phenomenon to interfere with the gene expression in various plant systems. A few examples are Petunia, Arabidopsis, Papaver, Torenia, Coffea and Rice. (Stam *et al.*, 1997; Chuang and Meyerowitz, 2000; Wesley *et al.*, 2001; Stoutjesdijk *et al.*, 2002; Allen *et al.*, 2004; Fukusaki *et al.*, 2004; Lee *et al.*, 2004; Ogita *et al.*, 2004; Miki *et al.*, 2005)

RNA interference (RNAi) is an evolutionarily conserved, ubiquitously distributed eukaryotic mechanism of sequence-specific transcription and translation inhibition of gene expression and regulates gene expression at chromatin level. In its functions RNAi serves as a cellular “Immune System” against invasive genetic elements such as viruses and transposons, as well as it works as a regulatory element of gene activity in the cell.

The basic principle of RNAi is the production of double stranded RNA (dsRNA) that can be detected by the cell system for degradation. This dsRNA may be in the form of viral replicating genome, transposone or any foreign element introduced to produce double stranded RNA. Artificial introduction of endogenous sense or antisense RNA strands into the cell also gives rise to the production of double stranded RNA leading to gene silencing. This is termed as co-suppression and antisense phenomenon while the introduction of both sense and antisense strands simultaneously is called RNAi. Co-suppression and antisense were identified first and were used for silencing of genes for function identification but later on it

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was reported that the use of RNAi for gene silencing is far more effective than co-suppression or antisense. (Fire *et al.*, 1998; Travella *et al.*, 2006)

RNAi can be used at the single cell as well as at tissue level by virus induced gene silencing (VIGS; Burch-Smith *et al.*, 2004; Scofield *et al.*, 2005; Hein *et al.*, 2005; Travella *et al.*, 2006) but this does not bring a stable genetic change and it is not possible to analyse the function of a gene at whole genome level using this transient approach. In order to analyse gene function at whole genome level the plants are transformed with RNAi constructs bringing the knock down in expression of the gene of interest exclusively in the plant.

The ease of its application for reverse genetics at the genome level makes it a powerful technique in gene function analysis. Gene constructs which encode intron spliced complementary RNA have been shown to induce 100% gene silencing at post transcriptional level when used against viruses, endogenous genes and transgenes (Smith *et al.*, 2000; Wesley *et al.*, 2001).

Wheat genome is a sum of three homeologous genomes (A, B and D) due to its hexaploid nature. In most of the cases each gene is present thrice in wheat genome (one copy belonging to each homeologous genome). These genes are expressed most of the times which gives rise to higher gene redundancy. This situation also creates hazards in the knock down of genes because some times it is not possible to knock down the expression of all the three copies of a gene present in the genome (Mochida *et al.*, 2003; Travella *et al.*, 2006).

Inspite of big size and hexaploid nature of wheat genome it has been shown that it is possible to deliver dsRNA into wheat genome and see the knock down effects transiently as well as by stable genetic transformation. A reduction in the VRN2 and VRN1 transcript was shown by RNAi which resulted in accelerated and delayed onset of flowering respectively. Although in both the cases the number of transgenic lines was not more than one. This technique has also been used to silence alpha gliadin genes of wheat by Becker *et al.*, 2006 and Humanes *et al.*, 2008 where they got complete knock down and 33%-80% reduction respectively.

Travella *et al.*, 2006 did a detailed study of *Phytoene Desaturase* (PDS) and *Ethylene Insensitive 2* (EIN2) knock down by RNAi (intron spliced) constructs. They got significant reduction to complete knock down of gene expression in 78 transgenic lines for PDS and 18 lines for EIN2 genes. They not only observed the reduction in gene expression but also the increase in siRNA complementary to the target genes. They found out that all the three copies of relevant target genes in hexaploid genome were knock down. They evaluated their knock down lines phenotypically as well as at molecular level at least until T₂ generation. From these results in wheat and the work of Richard *et al.*, 2003 with allotetraploid *Arabidopsis*

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suecica it can be concluded that use of hp-RNAi constructs for silencing of genes in polyploids is successful.

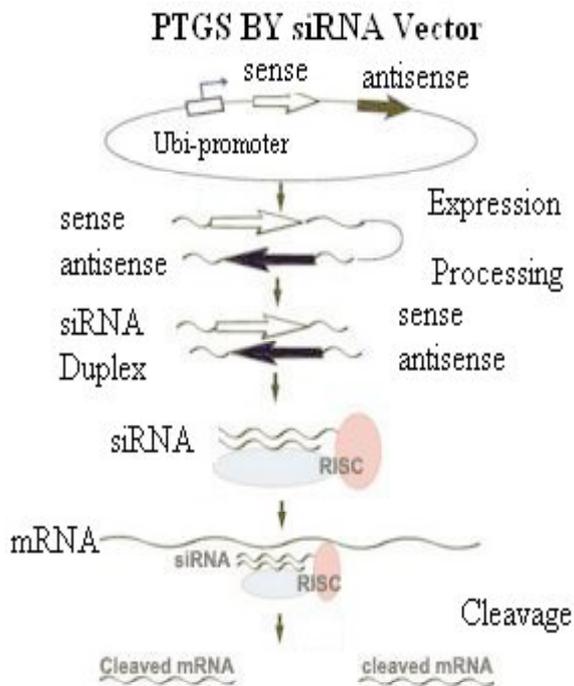


Figure 1.3

PTGS construct is made by cloning of small cDNA fragments (90-816 bps) from the target gene as an inverted repeat separated by a spacer region. Cassette is run by a promoter and stopped by terminator. Expression of construct produces same sequence of RNA in sense and antisense direction. Processing of mRNA leads to double stranded RNA which is identified by Dicer to cleave it into siRNA. These siRNA are then incorporated into RISC complex which identifies its substrate through its homology to siRNAs and targets equivalent mRNA for destruction.

Figure: 1.3. Mechanism of PTGS/RNA interference

1.5 Inducible Vs Constitutive Promoter In Disease Resistance:

Many early attempts to engineer plants by constitutive over expression with components of defence resulted in disease resistant but poor quality of plants. The reason was extensive cellular reprogramming of the defence components in the uninfected plant tissues that brought weak plants resulting in yield reduction. In comparison pathogen inducible promoters might bring reduction in the cost of resistance by expressing only in the area of infection. But the transgene must be expressed rapidly enough to block the growth and development of pathogen (Gurr and Rushton, 2005). Use of constitutive promoter does however suite some strategies not all, for example in Arabidopsis NPR1 over expression using CaMV 35S promoter brought broad spectrum resistance with normal phenotype while in maize same was done using *Ubiquitin* promoter and it gave rise to diseased genotype with out infection (Cao *et al.*, 1998; Piererse and Loon, 2004). A 42 kD endochitinase from *Tichoderma hematum* was over expressed in tobacco under the control of CaMV 35S promoter and *nos* terminator. CaMV is most widely used constitutive promoter in plants and is reported to be used in 80%

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of the transgenics. The resultant plants showed increased resistance to grey mold but the stem part was still susceptible. The same gene was then transformed using Actin-7 promoter from *Arabidopsis* which is auxin and wound inducible. The transgenic plants showed again resistance and this time in the stem as well. The reason might be the high copy number in case of inducible promoter (Kalai *et al.*, 2006 and Kalai *et al.*, 2008). From these results it can also be concluded that even for constitutive promoter it is not necessary to express uniformly through out the plants. While in inducible promoters the expression is controlled. Depending upon the results of this kind it was decided to use both constitutive as well as inducible promoters to over express *HarChit* and *HarCho* genes in the present studies.

1.6 Role Of Plant Callose Synthesis During Fungal Penetration:

Many fundamental processes have been explained at molecular level in plant biology but still some of them are discussed controversially. One such phenomenon is the role of callose in plant development and defence. Particular attention has been given to callosic papillae or cell wall thickenings formed in response to microbial attack. Callosic papillae were discovered at the site of fungal infection more than 140 years ago by deBary. Later Mangin reported in 1895 that papillae are formed of callose, a (1,3)- β -glucan with some (1,6)-branches (Aspinall and Kessler, 1957).

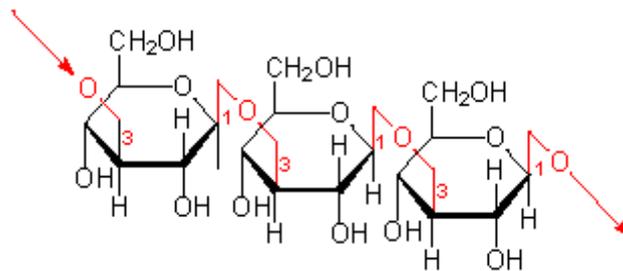


Figure: 1.4. Chemical structure of callose

Callose is a (1,3)- β -glucan composed of glucose units linked via (1,3)-glycosidic linkages. As a result, the polymer is arranged in a helix.

Continuous examinations have proved that callose is the most abundant chemical found in papillae (Aist and Williams, 1971; Sargent *et al.*, 1973; Mercer *et al.*, 1974, Sherwood and Vance, 1976; Mims *et al.*, 2000). Papillae are regarded as the physical barriers that do not stop pathogen completely rather slow down pathogen growth and in the mean time host plant initiates its defence reactions which require gene activation and expression. Defence reactions involve multiple pathways for the initiation of hypersensitive response, synthesis of phyto-

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alexins and production of pathogenesis proteins (PR; Lamb and Dixon, 1997; Brown *et al.*, 1998). Although the role of callose in plant defence has been investigated well over a century but its exact role is still to be fixed. Callose is predominantly present in papillae and the papilla is co-related with penetration failure but not with successful fungal establishment. Additionally callose is found at various places during normal plant growth e.g around pollen mother cells, in pollen grains and pollen tubes, at cell plates, plasmodesmata canals, root hair, spiral thickenings in tracheids as well as in the sieve plates of phloem elements. Additionally callose is induced at the site of wound and abiotic stress (Stone and Clarke, 1992; Kaus, 1996; Ryals *et al.*, 1996; Donofrio and Delaney, 2001; Jacobs *et al.*, 2003). Due to the involvement of callose in many important plant processes, efforts have been made to purify and characterise callose synthases and their corresponding genes from plants (Meikle *et al.*, 1991; Schlupmann *et al.*, 1993; Dhugga and Ray, 1994; Bulone *et al.*, 1995; Kudlicka *et al.*, 1995; Kudlicka and Brown, 1997; McCormack *et al.*, 1997; Turner *et al.*, 1998; Him *et al.*, 2001; Jacobs *et al.*, 2003, Voigt *et al.*, 2006). Although the purification of callose synthase enzyme to homogeneity is not achieved but molecular mass and subunit composition could be identified. Partially purified callose synthase have shown six to nine major polypeptides of 25 to 92 kD (Kamat *et al.*, 1992; Wasserman *et al.*, 1992; Dhugga and Ray, 1994; McCormack *et al.*, 1997). Although catalytic subunits are reported in between 32 to 57 kD some reports also showed catalytic activity associated with 200 kD polypeptide (Read and Delmer, 1987; Frost *et al.*, 1990; Delmer *et al.*, 1991; Li and Brown, 1993; Gibeaut and Carpita, 1994; Turner *et al.*, 1998; Hong *et al.*, 2001; Li *et al.*, 2003).

It is the structure of callose synthase that brings difficulties in its preparation. Its suggested structure seems to be multi-subunit and membrane associated enzyme complex (Verma and Hong, 2001, Figure 1.5). The detergents used in its extraction result in dissociation of complex and loss of activity. As purification of callose synthase to homogeneity is not possible therefore its activity could not be associated with the amino acid or nucleotide sequence.

1.6.1 Glucan Synthase-Like (GSL) Family Of Genes In Higher Plants:

Glucan synthase like (GSL) family of genes in plants is identified as genes encoding callose synthases (Cui *et al.*, 2001; Hong *et al.*, 2001; Doblin *et al.*, 2001; Østergaard *et al.*, 2002).

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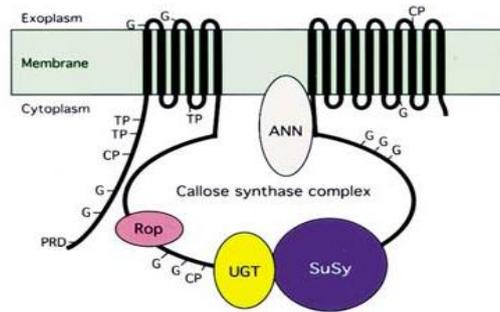


Figure: 1.5. Hypothetical model of callose.

Transmembrane domains and hydrophilic loop interact with Rho-like protein of plants (Rop), annexin (ANN), UDP-glucose transferase (UGT), and sucrose synthase (SuSy). G, potential N linked glycosylation sites; CP, cAMP- and cGMP-dependent phosphorylation sites; TP, potential tyrosine phosphorylation sites; PRD, proline-rich domain (after Verma and Hong, 2001).

The function associated to *GSL* genes is based on their homology with *FKS* genes in yeast where they are involved in callose synthesis. Six members of *GSL* gene family have been identified in barley and it is shown that the predicted amino acid sequence of *HvGSL1* correlates with the amino acid sequence of an active (1,3)- β -glucan. *HvGSL1* is relatively highly expressed in developing grains at early stage, coleoptile, florets and roots but not in fungal infected leaves (Li *et al.*, 2003). The most detailed analysis about the regulation and function of *GSL* genes is available in Arabidopsis. Twelve members of *GSL* gene family are identified in Arabidopsis (Richmond and Somerville, 2000; Verma and Hong, 2001) and the function of seven of them has been identified so far. *ATGSL6* encodes a callose synthase specific to cell plate and the transcript of this gene is slightly increased in the leaves when inoculated with *Blumeria graminis* spores (Jacobs *et al.*, 2003). *ATGSL5* and *ATGSL1* also show increase in transcript level when inoculated with *Blumeria graminis* spores. These genes also play an essential and redundant role during pollen development and fertility (Enns *et al.*, 2005). Functional analysis of *ATGSL8* and *ATGSL10* by gene disruption and RNAi showed an important role of both in male gametogenesis and plant development. It was found out that *ATGSL8* is essential for the entry of microspores into mitosis while *ATGSL10* mutants do enter mitosis but create abnormalities at cytokinesis in terms of symmetry and separation of tetrads. It was also seen that *GSL* family in general plays role in plant development. *ATGSL5* knock down were seen to have slightly stunted growth, later it was shown that knock downs of *ATGSL8* and *ATGSL10* in the background of *ATGSL5* knock down show increased growth abnormality. Plant with all three knock down genes showed the worst phenotype (Jacobs *et al.*, 2003; Töller *et al.*, 2008; Huang *et al.*, 2009). *ATGSL2* is seen to express itself

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predominantly in anthers but some transcript is also present in vascular bundles. It plays a role in exine formation during microgametogenesis and for pollen viability.

ATGSL5 contributes callose synthase to the infection papillae and is involved in the regulation of salicylic acid pathway (Jacobs *et al.*, 2003, Nishimura *et al.*, 2003). *ATGSL5* knock down plants showed complete resistance to the many virulent strains of *Perenospora parasitica* although callose was absent in the infection papillae (Nishimura *et al.*, 2003). This has raised questions to the classical model of resistance where callose seems to strengthen papillae against pathogen.

In wheat a family of eight TaGSL genes has been reported and an expression profile has been presented by Voigt *et al.*, 2006. They reported that the expression of these genes is organ specific in wheat. Three genes are selected in the present study from this family for the identification of any of their roles in disease resistance. *TaGSL10* is specifically expressed in stem, *TaGSL-3* in spikes and *TaGSL-8* is expressed in general in leaf, stem and spike at very high levels.

1.7 Disease Resistance Mechanisms In Plants:

Plants possess a surveillance system which is used by plants to recognise the attacking pathogens. Once pathogen/pathogens are recognised plant induces defence mechanisms against them. Among the many defence systems in plants often gene for gene interaction between resistance (R) and avirulence (avr) genes from plant and pathogen respectively is activated to control resistance (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001; Jones and Dangl, 2006). Recognition of avr gene product by plant R-gene coded proteins leads to the activation of hypersensitive response (HR) a type of programmed cell death that occur at or near the point of pathogen attack (Morel and Dangl, 1999; Heath, 2000). HR response is meant for the death of plant cells which later on stop the spread of pathogen to the other parts of the plant. R-gene mediated resistance is also meant to the activation of salicylic acid signalling pathway that mediates the induction of certain PR proteins. Many of the R genes have been identified and several of them encode receptor like proteins (Bent, 1996)

1.7.1 Chitinases And Other PR Proteins In Disease Response:

Wheat like other higher plants has innate defence mechanisms. These involve the activation of inducible defence responses which restrict the spread of pathogen in incompatible interactions leading to the system acquired resistance (SAR). Most of these proteins have antifungal activity and selectively target cellular components of the pathogen. These include

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chitinases, β -(1,3)-glucanases and thaumatin like proteins (TLP). Chitinases and glucanases target fungal cell wall while TLP affect the permeability of fungal membranes (Linthorst, 1991). There are reports where it is noted that PR proteins such as TLP, chitinases, glucanases, sormatin and ribosome inactivating proteins (RIP) can play a role in resistance against diseases (Bliffeld *et al.*, 1999; Oldach *et al.*, 2001; Bieri *et al.*, 2003; Balconi *et al.*, 2007). Simultaneous induction of three chitinase isoforms was observed in maize seeds on infection by the fungus *Fusarium moniliforme* (Cordero *et al.*, 1994). Several chitinases have been identified in wheat and their role against pathogen has been established. Botha *et al.*, 1998 reported 7 constitutively expressed and 3 pathogen induced chitinase isoforms in wheat. Singh *et al.*, 2007 isolated 33kD chitinase from wheat and expressed it in *e.coli* to prove its antifungal role *in vitro*.

1.7.2 Genetic Engineering For Disease Resistance:

Plant diseases are being controlled in a variety of ways since long. These include the use of better cultural practices (sowing of clean seeds and keeping the field clean), use of fungitoxic chemicals, biological control and the use of resistant cultivars. Cultural practices are effective only to some extent but do not give sustainable solution, use of chemicals is not human or environment friendly and biological control is not easy to manage. Development of resistant cultivars remains the only sustainable solution to control plant diseases. However, the resistance sources in wheat are limited. Any mutation in the pathogen renders the loss of resistance source. Genetic engineering provides an additional source of variation through which breeders can develop new resistance sources and introduce the resistance traits from across the phyla.

1.7.2.1 Role Of Genetic Engineering In Wheat Disease Resistance:

Several classes of genes can be used to enhance disease resistance in wheat. These genes can be basically selected from other plants and pathogens provided they have a putative role in plant defence. A group of defence response genes that encode proteins like β -(1,3)-glucanases, chitinases, TLPs, RIPs and thionins have been tried in wheat to develop resistance against fungal pathogens. In most of the cases over-expression of these genes resulted in enhanced fungal resistance (Muehlbauer and Bushnell, 2003). Recently enhanced resistance to the powdery mildew have been shown by Bliffeld *et al.*, 1999 and Bieri *et al.*, 2003 while Oldach *et al.*, 2001 showed resistance to powdery mildew as well as to leaf rust pathogens. Over expression of β -(1,3)-glucanases, TLP1, RIP, α -1-purothionin and *AtNPR1* showed the

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resistance against *F. graminearum* (Chen *et al.*, 1999; Makandar *et al.*, 2006; Balconi *et al.*, 2007; Mackintosh *et al.*, 2007; Shin *et al.*, 2008).

1.7.3 Targeting Fungal Cell Wall Polymers

Many antifungal proteins target fungal cell wall. This principle can be used as a strategy when designing genetic engineering projects for disease resistance. Chitin, chitosan, and β -(1,3) glucan are the structural polymers of cell wall in many fungi. Aminoglucan chitin (poly-GluNAc) is present widely in nature e.g in basidiomycetes, ascomycetes and phycmycete where it is a major component of fungal cell wall (Figure 1.6). Mucorales, in particular *Mucor*, *Absidia* and *Rhizopus* species contain chitosan (poly-GlcN). Some portion of chitin is always de-acetylated to form chitosan. Chitinases are enzymes which hydrolyse β -(1,4) linkages endolytically in chitin molecules (Cabib, 1987). These enzymes are found in nature including plants. Chitinases are important component of defence system in plants (Bartnicki-Garcia, 1968; Chen *et al.*, 1982; Jones *et al.*, 1986; Collinge *et al.*, 1993). In fungi they are important for cell division and differentiation and help saprophytic and mycoparasitic fungi to get their food (Papavizas, 1985; Cabib, 1987; Kuranda and Robin, 1991).

Keeping in view their lytic action against fungal cell wall, many chitinase genes have been cloned from bacteria, yeast, plants and filamentous fungi (Jones *et al.*, 1986; Watanabe *et al.*, 1990; 1992; Kuranda and Robbins, 1991; Yanai *et al.*, 1992; Blaisean and Lafay, 1992 Collinge *et al.*, 1993)

A chitinase gene cloned from *Serratia marcescens*, when overexpressed in *Trichoderma harzianum* remarkably increased the biocontrol ability of *Trichoderma harzianum* for *Sclerotium rolfsii* compared to wild type (Chet *et al.*, 1993)

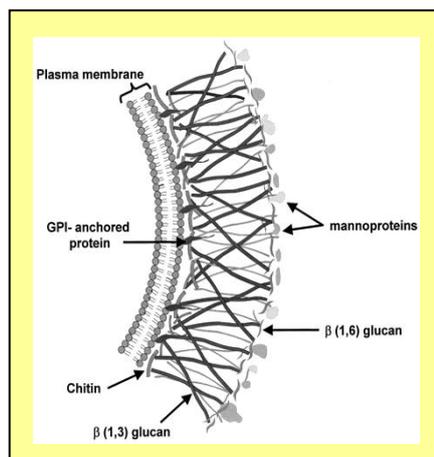


Figure 1.6: Structural components of fungal cell wall.

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1.7.4 *Trichoderma harzianum* Chitinases and Disease Resistance:

Saprophytic or mycoparasitic fungi are a group of fungi that parasitize other fungi as a source of their nutrition. *Trichoderma harzianum* is one of such fungus and has been used as a biocontrol agent for fungal plant pathogens (Papavizas, 1985). It produces the enzymes which destroy the structural components of fungal cell wall (Hendrix and Stewart, 2002). *Trichoderma* spp. have been used as biocontrol agents for many economically important plant pathogenic fungi such as *Botrytis*, *Rhizoctonia* and *Sclerotinia* (Steyaert *et al.*, 2004). A total of 16 genes have been found to be involved in mycoparasitism in *Trichoderma* spp. These genes have been cloned and sequenced (Kubicek and Penttila, 1998; Lorito, 1998; Cohen-Kupiec *et al.*, 1999; Donzelli *et al.*, 2001). A total of 5-7 distinct enzymes seem to be involved in biocontrol activity of *Trichoderma* spp. 2 of them are β -(1,4) acetylglucosaminidases (102 and 73 kD) and 4 are endochitinases (52, 42, 33, and 31 kD). A 42 kD endochitinase enzyme was isolated from *Trichoderma harzianum* and its *in vitro* activity was shown against *Botrytis cinerea*. It was found out that this enzyme can inhibit growth, spore germination and germ tube elongation of many fungi (Lorito *et al.*, 1998; Lorito *et al.*, 1994, Schirmböck *et al.*, 1994; Giczey *et al.*, 1998). It seems from these results that chitinases are the key enzymes used by *Trichoderma harzianum* for mycoparasitism (Ridout *et al.*, 1986; Chérif and Benhaman, 1990).

Similarly chitinase genes have been cloned and their products have been used in bioassays against many fungi of economic interest. A *Trichoderma* chitinase was cloned in *E. coli* and the transformed bacteria were used in irrigation to see the impact on *S. rolfisii*. The results showed a significant reduction in pathogen population in the irrigated field (Chet *et al.*, 1993). The purified enzymes from *Trichoderma harzianum* are highly effective against fungal pathogens. They are not only able to lyse the soft structures but also the hard structures like cell wall of mature hyphae, conidia, sclerotia and chlamydospores (Lorito *et al.*, 1998). When compared with the enzymes of plant origin, chitinases and (1,3)- β -glucanases of *Trichoderma* origin are 100 times stronger than their counterparts of plant origin. They are also non toxic to plant tissues even at very high concentrations (Lorito *et al.*, 1994; Lorito *et al.*, 1996) and their antifungal activity is enhanced synergistically when used in combination with PR proteins, fungicides or toxins and biocontrol bacteria (Lorito *et al.*, 1998; Steyaert *et al.*, 2004).

It has been demonstrated that the transformation of tobacco and potato with 42 kD endochitinase from *Trichoderma harzianum* improved resistance against *A. solani*, *Botrytis cinerea* and *Rhizoctonia solani* (Lorito *et al.*, 1998). In addition to antifungal activity

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chitinases from *T. harzianum* have also been shown to increase tolerance against salinity and heavy metals in transgenic tobacco (Dana *et al.*, 2006).

1.7.5 Chitosanase in Defence Response:

Chitosanase is an enzyme that can digest chitosan and therefore can be used as a potential candidate for slowing down the infection process by its capacity to degrade chitosan present in the fungal cell wall (Hendrix and Stewart, 2002). Additionally the oligomers produced by the chitosanases are relatively shorter than the oligomers produced by the chitinases after hydrolysis of chitin. These oligomers are used by the plant system to elicit defence responses such as stomatal closure and cell wall lignification. It has been observed that the smaller oligomers of fungal cell wall are stronger elicitors of defence than the larger oligomers (Vander *et al.*, 1998; Lee *et al.*, 1999).

A chitosanase gene was cloned from *Paenibacillus* sp. 61724 and transformed into tobacco. It was observed that defence response was activated rapidly in the transgenic tobacco plants compared to wild type tobacco plants (Hendrix *et al.*, 2001; Hendrix and Stewart, 2002).

1.7.6 Co-expression of *HarChit* and *HarCho* for Enhanced Defence

Plant defence systems can be supported by the introduction of antifungal genes such as chitinase and chitosanase into plant genome. It is most likely that these genes work independently and synergistically to support plant defence systems. In literature evidences have been presented where two or more enzymes have been shown to work synergistically *in vitro* and *in vivo* to enhance resistance against microbial pathogens. Terras *et al.* 1993 showed in *in vitro* that the amount of thionin required for the 50% antifungal activity of wheat is lowered by 2- to 73 fold when 2S albumins are combined with thionin. Jash *et al.*, 1995 showed the synergistic enhancement of antifungal activity by co-expressing barley class-II chitinase and 1,3- β -glucanase and class-I RIP in tobacco. Co-expression of chitinase and chitosanase can therefore be beneficial to plant defence against fungal pathogens.

In the present investigation wheat was co-transformed with chitinase (*HarChit*) and chitosanase (*HarCho*) genes from *T. harzianum* as a mean of developing disease resistant wheat.

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MATERIAL AND METHODS:

2.1 Materials:

2.1.1 Laboratory Chemicals and Consumables:

Purity grade “p.A.” chemicals and deionised water (Milli-Q Plus Water system, Millipore, Bedford, U.S.A.) were used for preparation of all solutions and media. As dictated by different requirements, solutions and media were autoclaved (20 min, 120°C, 2 x 10⁵ Pa) or filter sterilised (0.22 µm Millex® GP or Steritop™, Millipore, Bedford, U.S.A.). General laboratory chemicals were purchased from: AgrEvo (Düsseldorf, Germany), Applichem (Darmstadt, Germany), Biomol (Hamburg, Germany), Brand (Wertheim/Main, Germany), Duchefa (Harlem, The Netherlands), Fluka (Buchs, Germany), Merck-Schuchard (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Münich, Germany). Restriction endonucleases, dNTPs, DNA polymerases, DNA/RNA modifying enzymes and other molecular biology reagents were from Invitrogen (Karlsruhe, Germany), Fermentas Life Sciences (St. Leon-Rot, Germany) and New England BioLabs (NEB, Frankfurt/Main, Germany). Gel-blotting papers were from Schleicher & Schüll (Dassel) and photographic papers for agarose gels were from Mitsubishi (Kyoto, Japan).

2.1.2 Plasmids:

Following plasmids vectors were used for the cloning of PCR products.

- i. pGEM-T (Promega, Germany)
- ii. TOPO-T (Invitrogen, Germany)
- iii. TOPO-Blunt (Invitrogen, Germany)

These plasmids contain multiple cloning (mcs) sites with rare enzymes to separate the fragment later using one or more enzymes from mcs. Additionally, *ampiciline* / *canamycin* resistance genes as well as β -galactosidase enzyme coding gene, the disruption of which facilitates selection of right clones are integrated.

1.1.2.1 Plant transformation Vector Back Bone:

pBluescript® II KS⁺ (Stratagene, La Jolla, USA, Short *et al.*, 1998) was used as a back bone for the cloning of plant transformation cassettes. *i.e.* RNAi vectors and over expression cassettes.

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2.1.3 Plant Material:

An old fashioned German variety 'Florida' was used in the transformation experiments. This variety is already reported to be transformable by Becker *et al.* 1994. The response of wheat *Fusarium graminearum* was studied by using again wheat 'Florida'. All the plant material was grown and maintained as stated by Serazetdinova, 2001 at the green house of department of "Applied Molecular Biology of Plants", University of Hamburg, Germany. Two weeks old plantlets of winter wheat were transferred to vernalization chamber (02°C, 4000 lux light for 9h) for the next 08 weeks. After vernalisation plants were brought back to green house (18°C /14°C day/night, 16 h light of 23000-25000lux) and were fertilized after 08 weeks with Gabi-hydro culture fertilizer (4N : 2P : 5K : 0.5MgSO₄: trace elements, Gabi Biochemicals, Bad Salzuflen).

2.1.4 Bacteria and Bacterial Culture medium

Chemically competent *E. coli* genotype Top10' (Invitrogen, Germany) were used for all kind of plasmid propagation. LB medium was always used to propagate the bacteria. Antibiotics *Ampicilin* and *canamycin* were used at the rate of 100µg/ml and 50µg/ml respectively depending upon resistance against in the plasmid (Sambrook *et al.*, 1989).

2.1.5 Fungal Pathogens:

Phytopathological examination of the transgenic plants was done by Powdery Mildew Pathogen *Erysiphe graminis* f.sp. *Tritici* (biotrophic fungus) and *Fusarium graminearum* (Moensch), the causal organism of Fusarium head blight of wheat. Single spore isolate of *erysiphe* named C17A was brought from Dr. Kerstin Flath (Federal Biological Institute for Plant Protection in Agriculture and Pasture Field, Kleinmachnow, Germany). The *gfp* labeled and non labeled *Fusarium graminearum* strain 8/1 was obtained Via W. Schaeffer (MPPG, Hamburg) from T. Miedaner (Miedaner *et al.*, 2000) and maintained on CM agar plates (Nirenberg, 1981) at 28°C. Induction of conidiation was performed by placing a mycelium plug on SNA-plates and incubating them for 2 weeks at 18°C under near-UV light (TLD 36 W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips) with a 12-h photoperiod. Permanent cultures were prepared as conidia suspensions in water and stored at -70°C.

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2.1.6 Partical Gun

Partical gun PDS 1000/HE (BioRad, Munich, Germany) was used for the delivery of DNA into wheat cells. Assoceries such as Bursting Disk, stop-net and Macrocarriers to be used in transformation were also purchased from BioRad. Gold particals of 0.4-1.2 μm (Hereaus Feinchemicals, Karlsruhe, Germany) diameter were used as the micro-carriers. For the creation of gas pressure necessary for transformation, Helium with purity grad 5.0 was used. This was purchased from company Meser- Griesheim (Hamburg, Germany).

2.1.7 Settling tower:

A settling tower was used for the inoculation of powdery mildew spores. The settling tower consists of a box with width, depth and height of 50 cm each. A cylinder of 5 litre volume was mounted at the top of the box and was connected with the latter with 10 holes and each hole was 10 mm wide. A horizontal tube with many perforations of 1mm connected with He gas cylinder containing a pressure of more than 5000 psi. This tower was arranged by Oldach *et al.*, 2001 in the department and was the idea of Aslam and Schwarzbach, 1980.

2.2 Methods:

2.2.1 Experimental design and scope of experiments:

The objective of this project was to find out potential candidate genes those can be used to enhance the resistance of wheat against an array of fungal pathogens, transformation of two antifungal genes from *Trichoderma harzeanum* into wheat and identification of disease inducible genes that can latter be used in sequencing a disease inducible promoter that can be used in future transformation experiments. For this purpose the project was completed in following parts.

- i. Over expression of fungal cell wall hydrolytic enzymes (Chitinase and Chitosanase) genes from *Trichoderma harzianum* into wheat.
- ii. Knock down of three members of *Glucan Synthase Like (GSL)* gene family of wheat by post transcriptional gene silencing (PTGS).
- iii. Identification of genes in wheat related cereals those are shown to be up regulated in micro array study of *F. graminearum* infection and Practical verification of this up regulation.
- iv. Transgenic plants with knock down *GSL* genes and the tranogenics with over expressed fungal cell wall hydrolytic enzymes were evaluated against biotrophic

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fungus *Erysiphe graminis* f.sp. *tritici* and the necrotrophic fungus *Fusarium graminearum*.

Wheat genotype Florida was selected for transformation to get knock down of genes and over expression. This genotype is no more grown in the field but has been reported to be successfully transformable with high regeneration ability (Becker *et al.*, 1994 and Oldach *et al.*, 2000)

2.2.2 Sequence analysis:

All the sequence analysis *e.g.* sequence map analysis for cutting sites, alignment of sequences, selection of primer sequences was done mainly by computer programme DNA-Star (DNA Star Pvt, USA)

2.2.3 Cloning of Transformation Vectors:

Two different kind of transformation vectors were cloned depending upon the objectives. i). silencing of the three members of Glucan Synthase Like gene Family of wheat and ii) over-expression of chitinase and chitosanase from *T. harzianum* into wheat.

2.2.3.1 Cloning of Silencing Vectors:

An *RNAi* master construct was cloned by joining *RNAi* cassette from *pLNU-GA* into the *Sfi-I* restriction sites of *pBlueSfi-BA* which provided the back bone to *RNAi* cassette. *RNAi* cassette contained constitutively expressed *ubiquitin-1(Ubi-1)* promotor from maiz (Christensen *et al.*, 1992) and nopalyn synthase terminator (*Tnos*) from *Agrobacterium tumefaciens* separated by a *gus* gene fragment as spacer with multiple cloning sites at both ends of the spacer.

2.2.3.1.1 *pRNAi-GSL-3*: The purpose of this construct was the silencing of *Ta-GSL3* gene of *GSL* family of wheat. A selected sequence of 152 bp (on transcription: strands of hairpin forming sequence) from *Ta-GSL3* was cloned in *RNAi* cassette in sense and antisense orientation separated by spacer region. The criterion for the selection of siRNA forming sequence was to select sequence with least similarity to the rest of the genes in the family in wheat and other cereals. (Kusaba, 2004)

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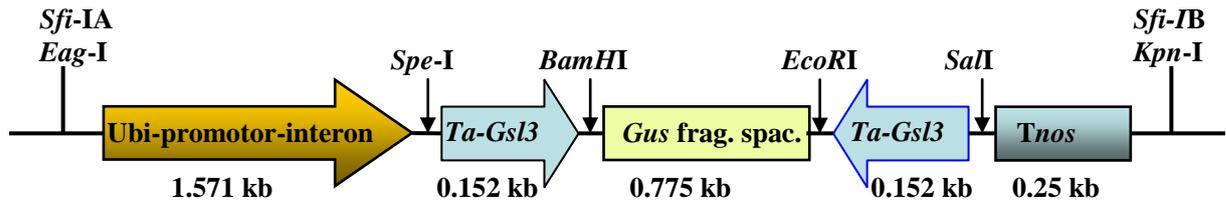


Figure. 2.1. pRNAi-GSL-3 Vector showing *ubi-1* promoter with *ubi* intron, *Gus* spacer, gene fragments and *Tnos* terminator as well as the cloning sites and the restriction enzymes used for cloning and Southern Blot analysis.

2.2.3.1.2 *pRNAi-GSL-8* and *pRNAi-GSL- 8'*: Both of these vectors were made to silence *Ta-Gsl8* and were tried to transform separately into wheat. The vectors were made by cloning siRNA forming fragments into the multiple cloning sites of master vector on both sides of spacer in sense and anti-sense orientation. The reason of making two separate vectors for the same gene was to see the effect of the size of siRNA producing fragment on gene silencing. In *pRNAi-GSL8* a fragment of 122 bp was used while in *pRNAi-GSL8'* a fragment of 230 bp was used.

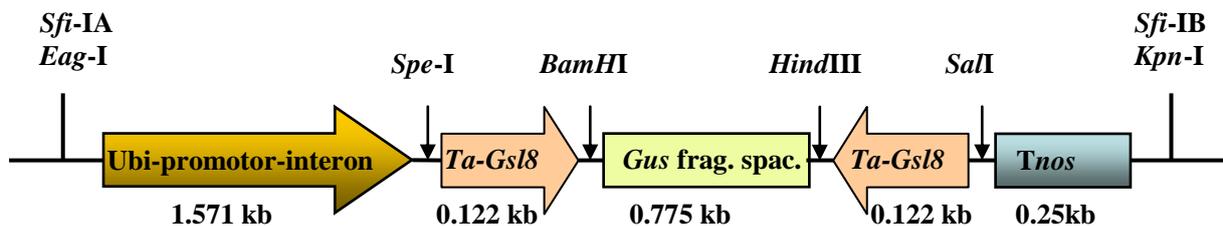


Figure: 2.2a. *pRNAi-GSL8* vector showing *ubi-1* promoter with intron, *Gus* spacer, siRNA forming gene fragments (122 bp) and *nos* terminator as well as the cloning sites and the restriction enzymes used for cloning and Southern analysis.

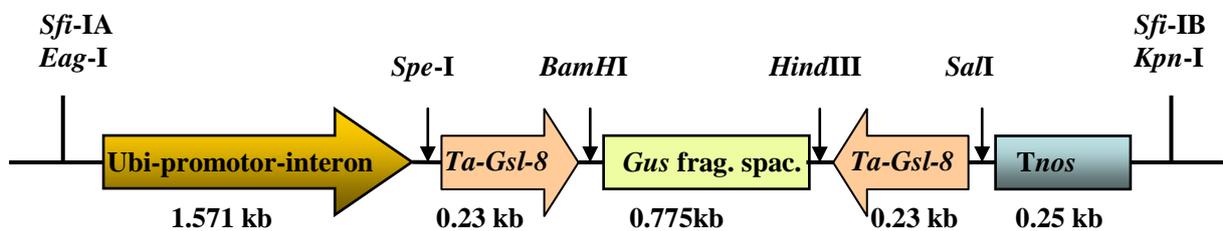


Figure: 2.2b. pRNAi-GSL8' vector showing *ubi-1* promoter with *ubi* intron, *Gus* spacer, siRNA forming gene fragments (230bp) and *nos* terminator as well as the cloning sites and the restriction enzymes used for cloning and Southern Blot analysis

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2.2.3.1.3 *pRNAi-GSL-10*: This vector was made to silence the *Ta-GSL10* gene of *GSL* family. A fragment of 230 bp was cloned on both sides of spacer in sense and antisense orientation.

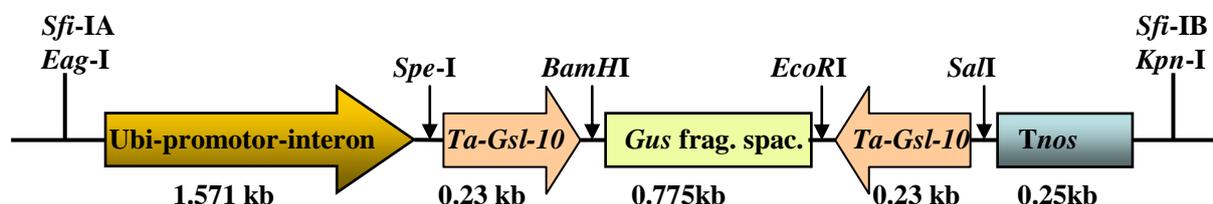


Figure: 2.3. *pRNAi-GSL-10* vector showing *ubi-1* promoter with *ubi*-intron spacer, siRNA forming gene fragments (230bp) and *Tnos* terminator as well as the cloning sites and the restriction enzymes used for cloning and Southern analysis

2.1 Primers used for the amplification of short DNA sequences used in PTGS.

Primer name	Primer Sequence (5'...3')	Length
AH-GSL-3F1	CTGACTAGTGAGCGACTGGACG	22
AH-GSL-3R1	CTCGGATCCACGTCTGTCCAATA	24
AH-GSL-3F2	CTGAATGTCGACCGACTGGACG	22
AH-GSL-3R2	CTCGAATTCCCGTCTGTCCAATA	24
AH-GSL-8F1	TGGACTAGTGAAAGGAGAAGGG	22
AH-GSL-8R1	GGGGATCCATTCTTAACGCTCTC	23
AH-GSL-8F2	TGGAGTCGACAAAGGAGAAGGG	22
AH-GSL-8R2	GGGAATTCATTCTTAACGCTCTC	23
AH-GSL-8F	TGAAGCACGCGCACAGGC	18
AH-GSL-8R	GCATGGTTTTGATTTTCGGGT	21
AH-GSL10F1	CACTAGTTACTGGGCTTCTTTCC	23
AH-GSL10R1	TCGCTGGATCCTTCACGGGTC	21
AH-GSL10F2	CAGTCGACACTGGGCTTCTTTCC	23
AH-GSL10R2	CGCTCTGAATTCACGGGTCC	20
AH-GSL10F	GGCACGAGGCTTCATTCAGAT	21
AH-GSL10R	GCAACACGAAGAGCTGGATACC	22
AH-GSL8SU	TGACTAGTGCGCACAGGCTG	20
AH-GSL8SL	CGGGATCCTTCCATGGATGTCAGC	24
AH-GSL8ASU	TGAGTCGACCGCACAGGCTG	20
AH-GSL8ASL	CGAATTCTTTCCATGGATGTCAGC	24

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AH-GSL10SU	GCACTAGTGCCCACCCATC	19
AH-GSL10SL	CGGGATCCTGATGAAACACTTC	22
AH-GSL10ASU	GCTGTGCGACCCCACCCATC	19
AH-GSL10ASL	CGAATTCGTGATGAAACACTTCC	23

2.2.3.2 Cloning of over expression vectors:

Chitinase (*HarChit*) and chitosanase (*HarCho*) genes of *T. harzianum* were cloned separately under the control of constitutive *Ubiquitin (Ubi-1)* promoter from Maize and disease/ stress inducible gene *Vst* promoter from *Vitis vinefera* L. This made a total of four constructs for over expression.

2.2.3.2.1 pUbi-HarChit:

This construct was a kind gift from Dr. K. Ayoo (University of Hamburg, Germany) who isolated an inducible 1.275 kb chitinase (*HarChit*) gene from the mycelia of *Trichoderma harzianum*. The gene was polymerized with primers containing *HincII* restriction sites-overhangs. The *HincII* fragment was cloned into the *pUbiCas* vector with the constitutive *ubiquitin1* promoter (*ubi1* promoter) from maize (Christensen *et al.*, 1992) and the *nopaline synthase* terminator (*Tnos*) from *Agrobacterium tumefaciens* (Figure 2.4). The constructed *pUbiHarChit* vector of 5.766 kb also carried *pUC-19* back bone for *beta lactamase* and *ampicilin* resistance selection markers.

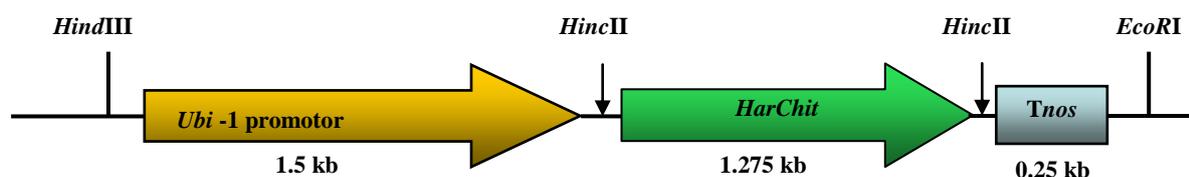


Figure: 2.4. *pUbi-HarChit*: vector showing *Ubi1* promoter, *HarChit* and *Tnos* terminator gene regions and restriction sites used in cloning and Southern Blot analysis.

2.2.3.2.2 pUbi-HarCho:

This construct again was cloned by Dr. K. Ayo (University of Hamburg). A 5.184 kb plasmid containing the *HarCho* gene was constructed by cloning the *BamHI/SacI* 708bp *HarCho* gene region from *T. harzianum* into the *pUbiCas* vector containing *ubi1* promoter, *Tnos* terminator and *pUC-19* back bone (Figure 2.5).

MATERIAL AND METHODS

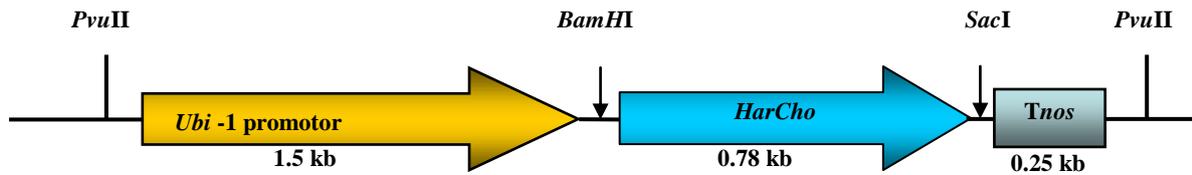


Figure: 2.5. *pUb-HarCho* vector showing *Ubi-1* promoter, *HarCho* and *Tnos* terminator gene regions and restriction sites used in cloning and Southern blot analysis

2.2.3.2.3 *pVst-Harchit*: This construct was cloned to express *Harchit* gene from *Trichoderma harzianum* into wheat under the disease and stress inducible promoter from *Vitis vinefera* L. (Leckband and Loerz, 1998). In order to enhance the transcriptional activity of the *Vst-1* promoter an enhancer fragment from the 35-S CaMV promoter was cloned 4 times at the 5' end of *Vst-1* promoter (Serazetdinova, *et al.*, 2005). *Tnos* was used as terminator. The constructed *pVst-HarChit* also carried pUC-19 back bone for selection markers.

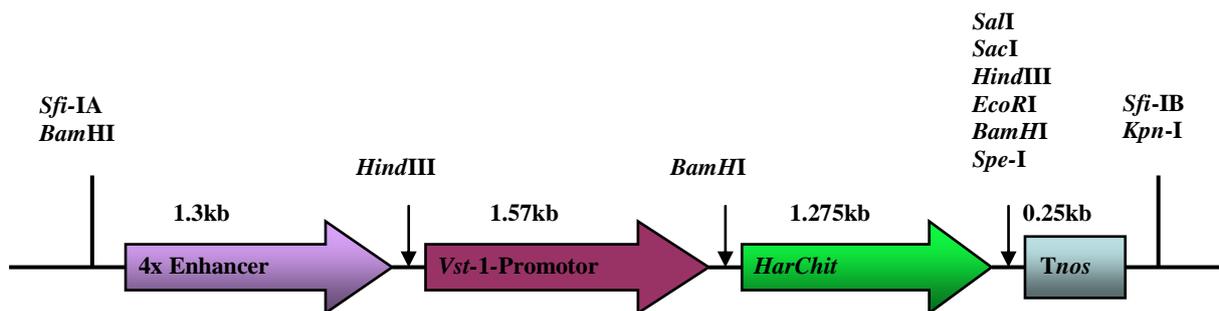


Figure 2.6 *pVstChit*: vector showing 4x enhancer, *vst-1* promoter, *Harchit* gene and *nos* terminator and the restriction sites used in cloning and enzymes used in Southern analysis.

2.2.3.2.4 *pVst-HarCho*: *HarCho* gene from *T. harzianum* was cloned under the stress and disease inducible promoter and 4x Enhancer of 35-S promoter of CaMV (Leckband and Loerz, 1998 and Serazetdinova, *et al.* 2005). *Tnos* from *A. bacterium* was used to terminate the gene transcription.

MATERIAL AND METHODS

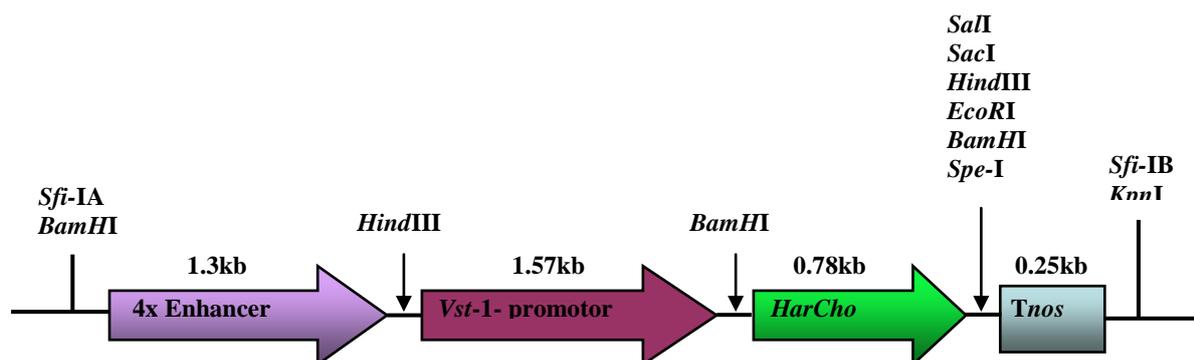


Figure: 2.7 *pVst-HarChit*: vector showing 4x enhancer, *Vst-1* promoter, *Harcho* gene and *Tnos* terminator and the restriction sites used in cloning and enzymes used in Southern analysis.

2.2 Primers used for over expression vectors:

2.2.1. Primers used for the amplification of Chitinase and Chitosanase genes:

Following primers were used for the amplification of full length chitinase and Chitosanase genes out of the plasmids *pUbiHarChit* and *pUbHariCho* mentioned above.

Primer name	Primer Sequence (5'...3')	Length(bp)	amplificate (bp)
Iqrar/Cho upper	GACTAGTCATGCGATCCAACGCTCTCCTTGGCCTCG	36	780
Iqrar/Cho lower	CGTCGACGCGTTAAGCCTTGAGGCCGGCAAC	31	
Iqrar/Chit upper	GACTAGTCATGTTGAGCTTCCTCGGAAAATCCGTAG	36	1275
Iqrar/Chit lower	GACGTCGACGCGTTAGTTCAGACCATTCTTGATGTT	36	

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2.2.2 Primers used for the Southern Blot analysis of Chitinase and Chitosanase genes:

Primer name	Primer Sequence (5'-3')	Length (bp)	Approx. amplificate (bp)
Chosouth-up/iqrar	CCTTCTCACTCCCCGCCAAC	20	200
Chosouth-low/iqrar	CGAAGACGACGTACGGGTGAAT	22	
Chitsouth-up/iqrar	CAACTGGGGCATCTACGACCG	21	200
Chitsouth-low/iqrar	GCTGCAGAAGGGAAGTTGGTGG	22	
Iqrar-cho400for	GACAACCACAAATCGGGAACG	21	400
Iqrar-cho400rev	CGTGGCCGTTATCACCGCT	19	
Iqrar-chit400for	GAGAAGAGAGCCAACGGATACGC	23	400
Iqrar-chit400rev	TCAATACCATCGAAACCCCAATC	23	

2.2.3.3 Cloning of reporter and selection marker gene:

2.2.3.3.1 *pUbiGus*: This construct was taken from Dr. Brettschneider (University of Hamburg). The only purpose was to use it as marker for checking the functioning of the procedures of transformation experiments.

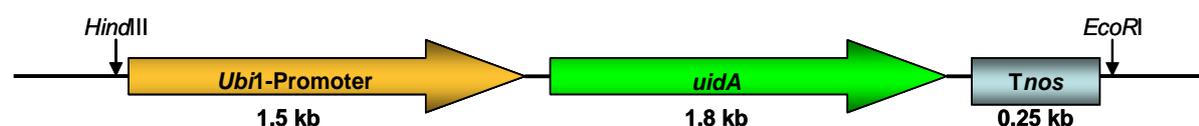


Figure 2.8 *pUbiGus* vector showing *ubi1* promoter, β -glucuronidase (*uidA*) and *Tnos* terminator gene regions and restriction sites used in cloning.

2.2.3.3.2 *pVstGus*: The construct was cloned to prove the functionality and infusibility of the cassette/promoter under transient expression. The β -glucuronidase *uidA* (Jefferson *et al.*, 1987) gene was cloned under 4x transcription 35-S enhancer, *Vst-1* promoter and *nos* terminator.

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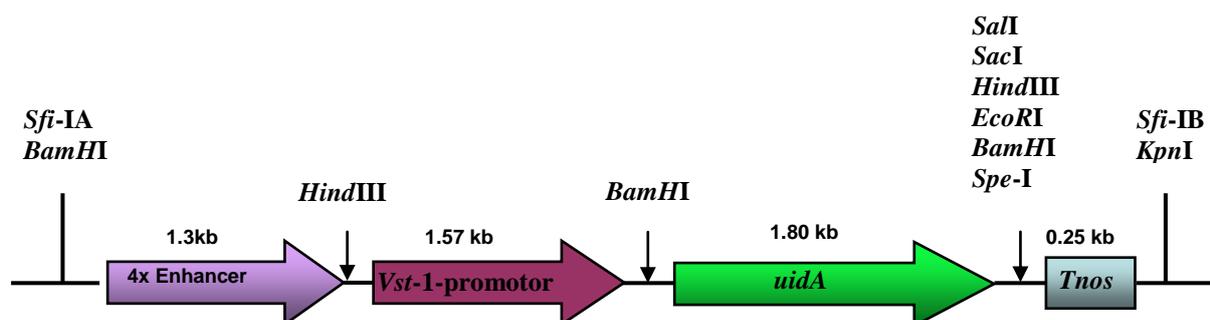


Figure 2.9 *pVstGus*: vector showing 4x enhancer, *Vst* promoter, β -glucuronidase *uidA* and *Tnos* terminator along with restriction sites used in cloning.

2.2.3.3.3 *P35SAcS*: This construct was used as a selection marker in transformation experiments. It contained a herbicide resistance creating gene *pat* (phosphinothricin acetyltransferase from *streptomyces viridochromogenes*, Strauch *et al.*, 1988) cloned under *35S Promoter* from cauliflower mosaic virus (CaMV) and *35S* (CaMV) terminator.

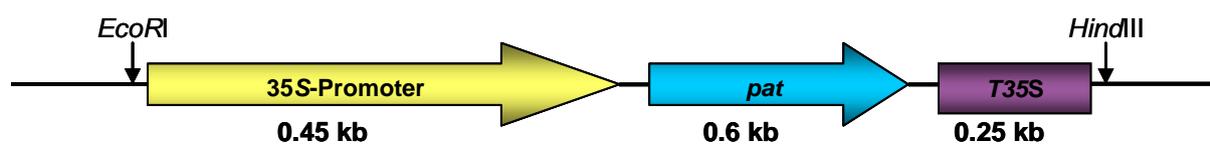


Figure 2.10 *P35SAcS* vector showing 35-S promoter, *pat* and T35S terminator gene regions and restriction sites used in cloning.

2.2.3 Primers used for the amplification of *pat* gene for Transgenic confirmation:

Primer name	Primer Sequence (5'....3')	Length
IQR/BAR-1	GAGACCAGTTGAGATTAGGCC	21
IQR/BAR-2	ATCTGGGTA ACTGGCCTAACT	21

2.2.4 Wheat Transformation:

Vectors with cloned gene cassettes were used to transform wheat using microprojectile bombardment of DNA coated gold particles on immature zygotic embryos (IZE). These bombarded IZE were then passed through a tissue culture procedure to get transgenic plants. (Becker *et al.*, 1994, Brettschneider *et al.*, 1997, Oldach *et al.*, 2001)

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2.2.4.1 Micro-projectile Bombardment:

Immature seeds were harvested 14-20 days after fertilization, washed 3 times with distilled water, sterilized with 1% Sodium hypochlorite containing 0.1% Mucosal for 25 minutes at room temperature on a shaker set at 200 rpm. The seeds were then rinsed 3 times with sterile double distilled water under the sterile hood. Immature embryos were thereafter aseptically isolated from the immature seeds using a dissecting microscope under standard sterile tissue culture conditions.

Gene delivery into the IZEs was carried out by particle bombardment using the BIORAD PDS-1000/He Particle Gun (BioRad, Munich, Germany). Isolated IZEs were cultured overnight on CIM and incubated on osmotic medium for 4 hours prior to bombardment. IZEs were bombarded with DNA-coated gold particles. DNA coating of gold particles and bombardment of scutellar tissue of wheat were based on the procedure described by Becker *et al.*, 1994. 2 mg gold particles with diameters of 0.4-0.8 μm were coated with 2 μg of plasmids DNA of each of the *pUbi-HarCho*, *pUbi-HarChit* and *p35SAcS* plasmids and suspended in 250 μl of absolute ethanol, from which 3.5 μl were loaded on to a macro-carrier and used to transform an average of 30 IZE (Appendix 1). The same procedure was repeated bomarding *pVst-HarChit*, *pVst-HarCho* and *p35SAcS*. While in case of gene silencing experiments, the constructs for *Ta-GSL3*, *Ta-GSL8* and *Ta-GSL10* genes were always used separately in combination with *p35SAcS*. In this case 2.5 μg of plasmid DNA was taken from each of both plasmids. For transient transformation with *gus*, 5 μg of the plasmid were used. Particle bombardment was carried out with Helium gas at 1350 psi under a partial vacuum of 27 mmHg according to Brettschneider *et al.*, 1997. The bombarded IZEs remained on the osmotic media for 18-24 hours before were transferred to CIM/SM-1.

2.2.4.2 Histochemical Gus assay

In order to check the efficiency of biolistic bombardment and related factors of transformation as well as the proper functioning of *Ubi-1* and *Vst-1* promoters some IZEs were bombarded with *pUbiGus* and *pVstGus* constructs cloned for the purpose. These IZEs were analysed for β -Glucuronidase (*Gus*) activity (Jefferson *et al.*, 1987) 48 hours after bombardment. IZEs were incubated for 12-16 hours at 37°C in staining buffer containing X-Gluc {(5-Bromo-4-chloro-3-indolyl- β -D-glucuronic acid); Sodium buffer (pH 7.0); 0.5% Triton X-100} as a substrate (Appendix 2). *Gus* signals were visually enumerated under a dissecting microscope.

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2.2.4.3 Tissue Culture of the Bombarded Embryos:

Isolated IZE were cultured in a regime of MS-based (Murashige and Skoog, 1962) Callus induction medium (CIM/SM-1), Selection Medium (SM-2), regeneration Medium (SM-3) and rooting medium ($\frac{1}{2}$ MS), with requisite macro elements, micro elements, recommended additives and plant hormones (Appendix 3). The media were solidified with 0.3% gelrite.

Tissue culture of the bombarded wheat IZEs was done according to Becker *et al.*, 1994, Brettschneider *et al.*, 1997 and Oldach *et al.*, 2001. Immature wheat zygotic embryos were used as *explant* source. Wheat plants were grown and maintained under standard green house conditions for winter wheat. 15 days old IZEs were isolated aseptically, bombarded with gene constructs of interest and *p35SAc5* construct that creates *BASTA* resistance. IZEs were transferred to CIM 18-24 hours after bombardment and were kept under dark at 26°C for the next two weeks. Callus induction medium contained 2mg/L 2, 4-D as auxin source for initiation and proliferation of calli while MS⁻ was used as nutrient source for plant material in all tissue culture media including CIM. SM-2 was the same CIM but 3 mg/L of herbicide *BASTA* was added to it so that only transgenic calli can be selected. Calli selection continued on SM-2 for two weeks in dark. After that calli were transferred to regeneration media (SM-3) which contained very low amount of 2, 4-D (0.1mg/mL) along with MS⁻ and 3mg/L *BASTA*. Calli were transferred to light at standard tissue culture conditions with 26°C temperature and 2000 lux light intensity for 16 hours per day. In 2-3 weeks already visible embryos started producing shoots and plantlets. Shoots and plantlets of 3-5 cm size were transferred to $\frac{1}{2}$ strength MS for proper plant development. In couple of more weeks plantlets were ready to transfer into the green house in normal soil conditions.

2.2.4.4 Selection and propagation of Transgenics

Regenerants were selected on the selection medium during tissue culture. High relative humidity was maintained in the green house for a week after the transfer of plants there. Two weeks old plants were then sprayed twice with 150mg/L and 200mg/L of *BASTA* herbicide respectively to make final transgene selection. The selected plants were then moved to vernalisation chamber for next two months and in the mean time transgenes were proved by molecular analysis. Vernalised plants were again maintained in the green house under standard conditions till maturity and the seeds were harvested to advance to the next generation.

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2.2.4.5 Segregation Ratio:

Segregation of the transgenes in progeny was evaluated from the expression of the herbicide resistance gene. T₁ seedlings were sprayed twice with 150 mg/L and 200 mg/L *BASTA* herbicide respectively 7 and 14 days after germination. Surviving and dead plants were then enumerated and computed to establish the segregation ratio.

2.2.5 Molecular analysis of putative transgenics:

The first step of transgenic analysis is to prove them at DNA level by PCR using primers specific to the transformed gene constructs. Second step is the Southern analysis. Although PCR is very sensitive but still there are many factors like quality of DNA that can affect the results. (Personal communication with Dr. Dirk Becker, University of Hamburg). Northern Blot analysis comes as third step that is used to check the functioning of integrated genes.

2.2.5.1 DNA Isolation

Genomic DNA was isolated by the protocols described by Palotta *et al.*, 2000. Approximately 200 mg of leaf material was taken in 2ml reaction tubes and frozen in liquid nitrogen before crushing by ball bearing through vigorous shaking using Retsch MM-2000 Shaker. Amplitude of 80 mm and motor speed of 200 rpm was set on Retsch. The milled powder was sequentially extracted with 800µL extraction buffer (appendix-4), 800µL Phenol/Chloroform/Isoamylalcohol (25:24:1) and centrifuged at 5000 rpm for 3 minutes. DNA was precipitated by treating the supernatant with 1/10 volume of 3M sodium acetate at 5.2 pH, 1 volume of isopropanol and by centrifuging at 1300 rpm respectively. The pellet was washed twice with 80% ethanol, air dried and dissolved in 100 µL R-40 (40 µg/ml RNase A in 1x TE, pH 8.2). Concentration, purity and integrity of isolated DNA were checked by absorbance spectrophotometry and gel electrophoresis. DNA was stored at 4°C before use.

2.2.5.2 RNA Isolation

Total RNA was extracted from the samples using the peqGOLD TriFast extraction protocol according to the manufacturer's instruction (PeqLab Biotechnology, Erlangen Germany) (Appendix-5). For Northern Blotting freshly prepared total RNA was used while for cDNA synthesis DNA-free RNA was generated by using an endonuclease DNase which digests single and double stranded DNA. Extracted 10 µg total-RNA was treated with 10 U of RNA-free DNase, appropriate buffer and RNase inhibitor as described by reagents manufacturers (Fermentas Life Sciences st Leon, Germany). The purity and integrity of the RNA was checked through gel electrophoresis and absorbance spectrophotometry. The DNA-free RNA

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was frozen in liquid nitrogen and stored at -70°C until used in reverse transcription for the generation of cDNA.

2.2.5.3 cDNA Synthesis:

cDNA was synthesized from the total RNA using 18-mer oligonucleotide (Oligo(dT)₁₈) primer, dNTPs, RNase inhibitor and the Moloney murine leukaemia virus reverse transcriptase (M-MuLV) as recommended by the reagents manufacturer (Fermentas Life Science, St. Leon Germany). Detailed procedure can be seen in appendix-6

2.2.5.4 PCR

PCR was always performed by making master mix of as many as possible common reagents. A detail of reagents is shown in appendix-7. Gradient PCR was always run to identify the best annealing temperature for every PCR reaction. A minimum of one minute extension time was used for the smallest expected product and was increased at the rate of one minute per kb of expected product in other reaction.

2.2.5.5 Southern Blot analysis:

Southern blotting was carried out as described by Sambrook *et al.*, (1989). 10-25 μg genomic DNA was restricted with the required endonucleases and separated in 0.8% agarose gels. Gel treatments for Southern blots were performed as described in Sambrook *et al.*, (1989) as well. DNA was transferred onto HybondTM NX nylon membranes by capillary transfer (20 x SSC) and fixed to membranes with 120 mJ using Stratalinker TM 1800 UV crosslinker (Stratagene, La Jolla, U.S.A.). Detection hybridisation with DIG-labelled DNA probes (20-25 ng/ml hybridisation solution) was performed at 42°C using DIG Easy Hyb solution (Roche, Mannheim, Germany). Chemiluminescence's detection was done with CSPD[®] substrate according to the manufacturer's prescriptions (Roche, Mannheim Germany). The detailed steps can be seen in the Appendix-8

2.2.5.6 Northern Blot analysis:

Freshly prepared RNA was run on the 1 percent degeneration agarose gel in 1X MEN buffer. RNase free conditions were assured by immersing gel combs and gel chambers at least for one hour in 3N NaOH solution. RNA was transferred to HybondTM N+ Nylon membrane by capillary transfer using 10 X SSC solutions and fixed to membrane with 1200J using Stratalinker TM 1800UV crosslinker (Stratagene, La Jolla, USA). For the detection

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hybridization positive single stranded DNA probes were radioactively labelled with P³² according to protocols given by manufacturers of DNA labelling kit (MBI Fermentas st. Leon-Rot, Germany). Detections were done on the X-ray films (Amersham, USA).

2.2.6 Phytopathology Experiments:

Inoculation of wheat transgenic and non transgenic Florida was done under controlled conditions in the infection chambers.

2.2.6.1 Inoculation with *Erysiphe graminis* f sp. *tritici*:

Inoculation studies with *Erysiphe graminis* f sp. *tritici* was performed on detached leaf segments.

2.2.6.1.1 Detached leaves infections:

In these experiments second leaf of two weeks old seedlings of T₁ transgenic (Both over expression and knock down lines) and control lines (wild type Florida as well as transgenic line containing *bar* gene only) were cut into 3 cm long segments. These segments were cultured on an anti-senescence media containing 0.4% agar, 10 ppm (parts per million) benzamidazole and 1ppm silver nitrate. It was made sure that both ends of the cut leaf be immersed into the medium. Cultured detached leaf plant material was incubated at least one hour under sterile conditions of clean hood before inoculation just for the acclimatisation of plant material. Culture plates of 20 x 10 cm² dimensions with small partitions of 5 x 3.5 cm² were used to culture detached leaves for inoculations. After the plant material had got familiarised with culture environment, inoculations were done in the settling tower (described in the materials). Freshly harvested powdery fungal material (mycelium/spore/conidia) was blown onto the cultured plant material and at least one hour was given to settle the fungus down onto the plates. It was made sure that at least 300-400 conidia should be available per cm². Conidia were counted by putting a scale in the settling tower among plates at the time of inoculation and later on conidia were counted under microscope. Culture plates were covered with lids and sealed with sealing film and kept at 16 °C in the dark for over night. After that these plates were transferred to infection chambers where a temperature of 18 °C and light to dark period ratio of 16:8 hours were maintained for the next 21 days.

For checking the performance of transgenics a data collection strategy was defined. After 9dpi the number of colonies was counted and at 21dpi the size of randomly selected colonies was measured and photographs were made to see the difference. For these experiments the

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protocols mentioned by Oldach *et al.*, 2001, Limpert *et al.*, 1988 and Kerstin Flath (personal communication) were modified to get good results.

2.2.6.1.2 Seedlings inoculations:

Two weeks old seedlings were sprayed with 0.5% aqueous tween-20 solution. One hour after spray, seedlings were infected in the settling tower by blowing the freshly harvested fungal material. When the fungal spores were settled down one hour after inoculation, the seedling trays were covered with transparent lids and stored in the dark for over night at 16°C and then transferred to infection chamber for next three weeks. In the infection chamber relative humidity of >80, temperature of 18°C and 16 hour light per day was maintained artificially.

2.2.6.2 Inoculations with *Fusarium graminearum*:

F. graminearum interaction with wheat is possible only at a specific stage of plant growth and it was done only on plant under controlled conditions.

The plants to be used in fusarium pathology were grown and maintained according to the procedures stated previously. Infection was performed at plants growing till anthesis (approximately 18-19 weeks after sowing of seeds in the soil). A single spike was inoculated with in lemma and palea of two basal florets of two central spikelets with a water droplet of 10µL containing 200 conidia. (Modified after Pritsch *et al.*, 2001 and voigt, 2005). The inoculated spikes were enclosed in small plastic bags during the first 3 days to ensure a high relative humidity for infection and to prevent a cross contamination of different *F. graminearum* isolates. In the infection chamber temperature of 18-20°C, relative humidity of >70 and photoperiod of 16 hours was maintained for the next three weeks. For the experiments leading to evaluation of transgenic lines data was collected on the visually effected kernels 21 dpi. Each transgenic line was inoculated at least twice and a maximum of 6 times and there were at least 40 spikes and 10 independently growing plants per line per experiment.

2.2.7 Identification of Disease Inducible Genes in wheat for Promoter sequencing:

This experiment was performed in three steps.

- I. As a first step literature was pearly reviewed for the identification of genes those are differentially expressed under the attack of *Fusarium graminearum* after different time points.
- II. Wheat genotype Florida was inoculated as a second step with *F. graminearum* and the expressivity of the selected genes was evaluated experimentally using reverse transcriptase

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PCR (rt-PCR) and Northern Blot analysis using radioactively labelled fragments specific to selected genes. Wheat inoculations were done under standard conditions using the protocols mentioned in 2.2.6.2. Infections were maintained only from 12hai to 144hai. Mock inoculations were done with water as a control. Inoculated and mock inoculated plant materials were harvested after different time points starting from 12 hours after inoculation (hai), 24 hai, and 48hai going to 144 hai. Each experiment was repeated thrice and there were three biological repeats in each experiment.

RNA was isolated and cDNA was synthesized. The primers specific to the genes selected from the literature review were designed and PCR was performed on inoculated and mock inoculated samples. Primers specific to the gene controlling synthesis of 18-S RNA (House keeping gene) were used as control for normal RNA isolation in all samples. The primers specific to all selected genes are given in table 2.3

2.3 Primers used in the Wheat Fusarium interaction studies for promoter identification

No	Name	Sequence (5'-3')	Gene
1	AH-INF-01F	TGGCGGCTACGATGACTGTG	Chalcon synthase
2	AH-INF-01R	GCTGTGACTGGGACGCTATG	
3	AH-INF-02F	CGTGGTGGAGCGAACAGTCT	PDR Like ABC transporter gene
4	AH-INF-02R	TGCCTCCATTA ACTCGTCAG	
5	AH-INF-03F	CCACGCTTGGCTGTGTATGC	PK0023
6	AH-INF-03R	GATACTGCTCGTGGTCTTCTTG	
7	AH-INF-04F	GCCTGTTTGCCACGCTGCTC	Oxalate oxidase precursor
8	AH-INF-04R	GCGGCAA ACTTGGACTTCAG	
9	AH-INF-05F	CATTATCTCGCAGTCGCTCTTC	Chitinase ning 7840
10	AH-INF-05R	GAGCTCTATCGAAACGCCATTG	

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11	AH-INF-06F	CGTTCCTCGCTCAGACTTCCC	Chitinase-3
12	AH-INF-06R	GATCGCACCATTATTCCCTTG	
13	AH-INF-07F	GACCATGTCCGACTGTGCCG	Cytochrome P-450
14	AH-INF-07R	GTGCGCAACATAACTAACTCCAG	
15	AH-INF-08F	CGAGGACGTGGAGGAGAACC	Glutathion-S-transferase
16	AH-INF-08R	TTCGGAGCAAATGAATGTATCT	
17	AH-INF-09F	CATCAAGAACAACCTGCCCTAC	Thaumatococcus protein-1
18	AH-INF-09R	GCACGCACGGACAGCCTTAT	
19	AH-INF-10F	CTCGAGCATAGCAACAGACT	Thaumatococcus protein-2
20	AH-INF-10R	CGTACGCCTTTTATTGATTCGC	
21	AH-INF-11F	GGCTTTGCTCTTGTTTGCTT	BM135610
22	AH-INF-11R	ATCAAAGGAGAATAAAAGAACAAG	
23	AH-INF-12F	CGCCGCCGCTCACATCC	PR-wheat
24	AH-INF-12R	GGGTCGGTGAGGATGAGCAG	

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3. RESULTS:

Wheat is attacked by a large number of fungal species which cause significant reductions in the grain yield and quality. The aim of this project was to find out the solutions for fungal diseases of wheat. In conventional plant breeding projects the development of disease resistant wheat varieties is one of the top priorities. The development of the wheat variety that is resistant to all of or most of the fungal species which attack wheat in the field is still not realised. Some varieties are developed which can resist against a few diseases but not against others the resistance is not normally stable and is broken after a few years. The dire need of the time is to develop wheat genotypes which can resist against some if not all the fungal diseases.

With the emergence and development of molecular biology, the scientists now are studying the phenomenon of disease resistance and pathogen virulence at molecular level. We know something now about the factors which help pathogen to attack and parasitize the plant. Similarly we know about the factors which can be used by plants against pathogens. We also know about the pathogens of the pathogens and these pathogens are being used as tools in biological controls. All these attributes of plants and the pathogens are controlled by genes and the manipulation of these genes by genetic transformation can be used for increasing resistance of plants against diseases.

In this project, two antifungal genes *HarChit* and *HarCho* from *Trichoderma harzianum* (myco-parasite) were co-expressed under constitutive *Ubiquitin* promoter as well as under stress and disease inducible *Vst-1* promoter in wheat. These genes hydrolyse the chitin present in the fungal cell wall. Inducible and constitutive promoters were used to get advantages of both the promoters and compare the effect on the disease resistance. To evaluate any of their effect on general disease resistance two fungal species with different mode of infection were used in infection assays.

Callose is a component of fungal cell wall and is found in various plant organs. This is also produced at the site of wounding and fungal penetration. Recently, gene families have been identified in some plants which control callose production. It was decided to find out the function of three members of this gene family from wheat in disease resistance, if any.

As a matter of the need it was decided to find out some genes in wheat those are differentially expressed under fungal attack. The objective was to use these genes in future experiments for the identification of their promoters and promoter of one or more of these genes can be used as disease inducible promoters in wheat transformation experiments because of the need of a perfect disease inducible promoter in wheat was direly felt in this project.

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3.1 Wheat transformation:

All the transformation experiments were done in already reported efficiently regenerating transformable wheat genotype Florida. The protocols used for transformation were mainly from Becker *et al.*, 1994 and Brettschneider *et al.* 1997.

In order to check if all the steps in transformation protocols are done rightly some IZEs had always been shot with *pUbi-gus* and *pVst-gus* constructs which contain *gus* reporter gene. Histology was done with these IZEs forty eight hour after shooting. Only those experiments were forwarded to tissue culture where the results were comparable to already stated ones. These results also confirmed the functionality of the promoters used in the transformation experiments.

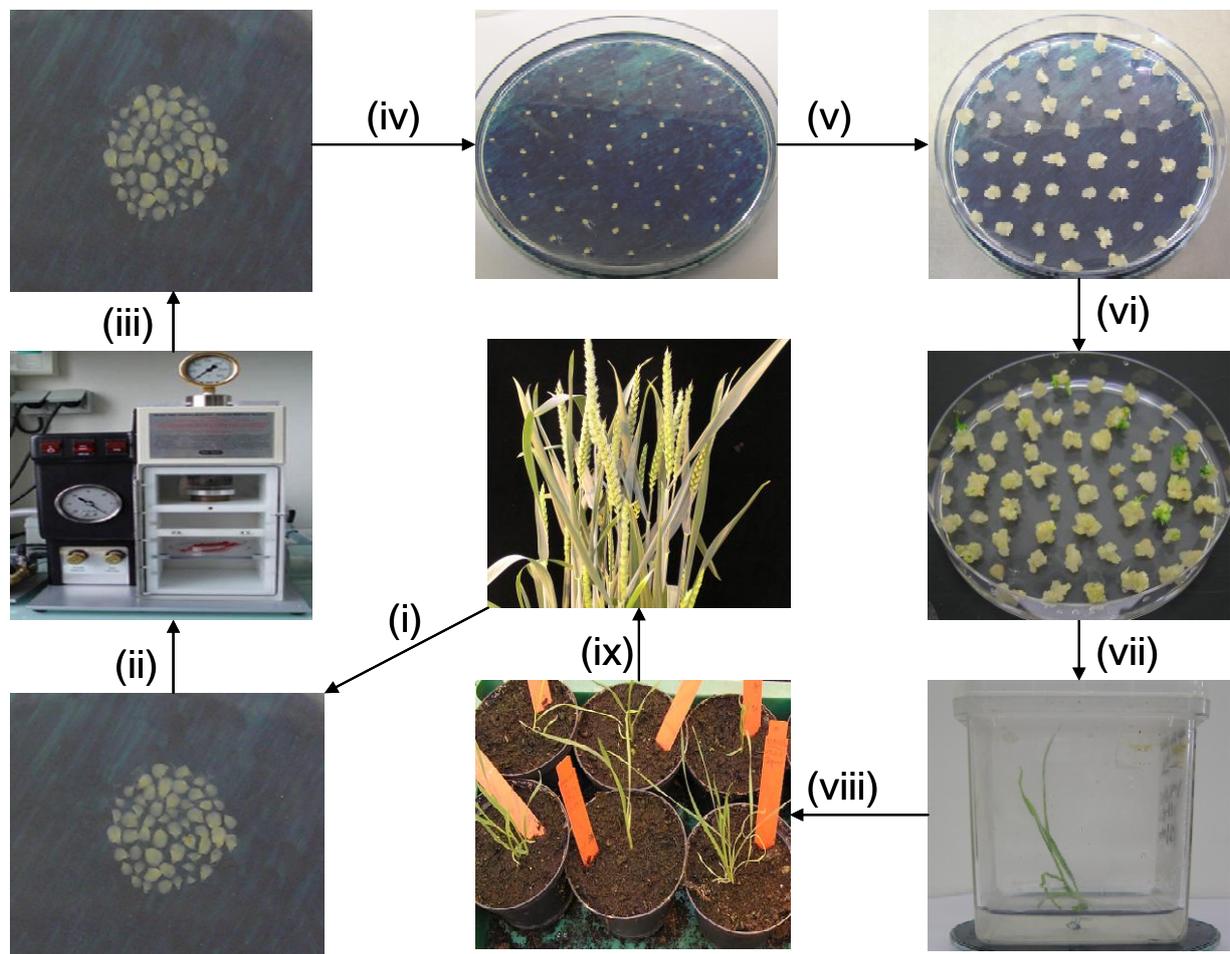


Figure: 3.1 General procedures for wheat Transformation:

- i) Immature embryos were isolated aseptically from wheat spikes two weeks after anthesis and stored on osmotic media before bombardment through Biolistic gene gun.
- ii) Bombardment is done with the gene of interest and selection marker gene
- iii) storage on osmotic media over night.
- iv) CIM is given for two weeks keeping in the dark.
- v) SM-II is given for two weeks by keeping in the dark.
- vi) SM-III / Regeneration media is given by keeping calli in

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the light conditions. vii) Developing embryos are transferred to rooting media for proper root and plantlet development. viii) five inches tall plantlets are transferred to green house and final selection is made there with selection agent. ix) Finally selected plants are kept in the green house and let them to reach maturity.

3.1.1 Over Expression *HarChit* and *HarCho* Genes in Wheat:

The details of the gene constructs for *HarChit* and *HarCho* are given in the section 2.2.3.1 IZEs were bombarded with these constructs and selection marker gene construct. Plants resistant to selection marker agent *BASTA* were selected. As shown in table 3.1 a total of 3128 IZEs were bombarded with *pUbi-HarChit* and *Ubi-HarCho* plus *P35SAcS* (selection marker gene construct) and 34 *BASTA* resistant plants were selected. Out of these 34 plants only 8 were proved to have the genes of interest (See figures 3.7 and 3.8). 3540 IZE's were bombarded with *pVst-HarChit* and *Vst-HarCho* plus *P35SAcS* and 31 *BASTA* resistant plants were recovered out of which only 6 were proved to contain the genes of interest.

Table: 3.1 Transformation frequency for the over expression of *Harchit* and *Harcho*

Gene constructs	No. of IZEs shoot	Plants recovered resistant to <i>BASTA</i>	Transgenic Plants with genes of interest	Transformation percentage for the selection Marker gene	Transformation percentage for the genes of interest
<i>pUbi-HarChit+Ubi-HarCho</i>	3128	34	8	1.08	0.26
<i>pVst-HarChit+Vst-HarCho</i>	3540	31	6	0.87	0.17

3.1.1.1 Transformation with *pUbi-HarChit* and *pUbi-HarCho*:

IZEs were isolated aseptically from spikes two weeks after fertilisation. IZEs were cultured over night on callus induction media and then for 4 hours on osmotic media before bombarding gold particles coated with a mixture of *pUbi-Harchit*, *pUbi-Harcho* and *p35SAcS* gene constructs. Following bombardment IZEs were maintained on SM-I (callus induction media without *BASTA* selection) for two weeks under dark at 26°C, SM-II (callus induction media with 3mg/L *BASTA* selection pressure) for two weeks again under dark at 26°C and SM-III (regeneration media) for two weeks under 2000 lux light at 26°C and after that developing somatic embryos were transferred to ½ MS media (rooting media) while some

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green calli were again transferred to SM-III. It took on an average of 2.5 months to get *BASTA* resistant regenerants from bombarded IZEs. These regenerants were transferred to green house where a final selection was made by spraying the plants with *BASTA* herbicide twice at an interval of one week after the plants got hardened there.

A total of thirty four plants were selected for the co-integration of *pUbi-Harchit* and *pUbi-Harcho*. The details for *pUbi-Harchit* and *pUbi-Harcho* are shown in figure 3.2 and 3.3.

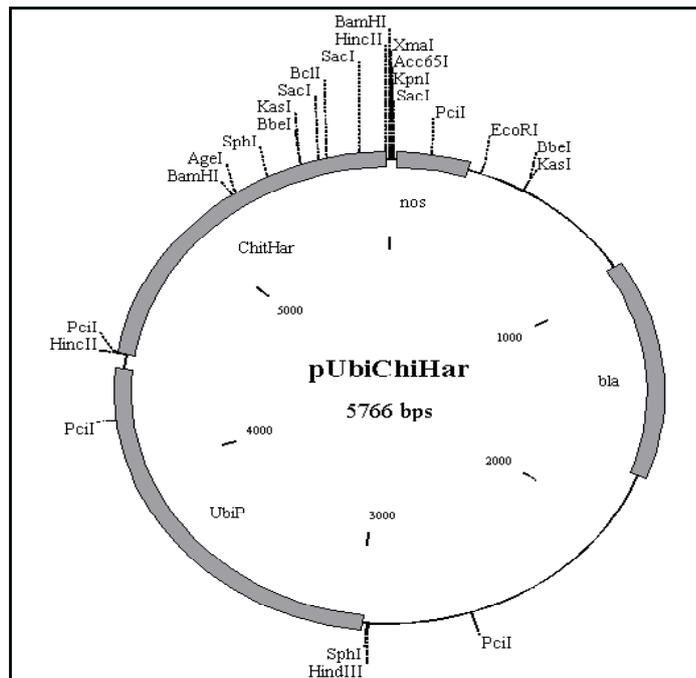


Figure: 3.2 Construct card for pUbi-HarChit

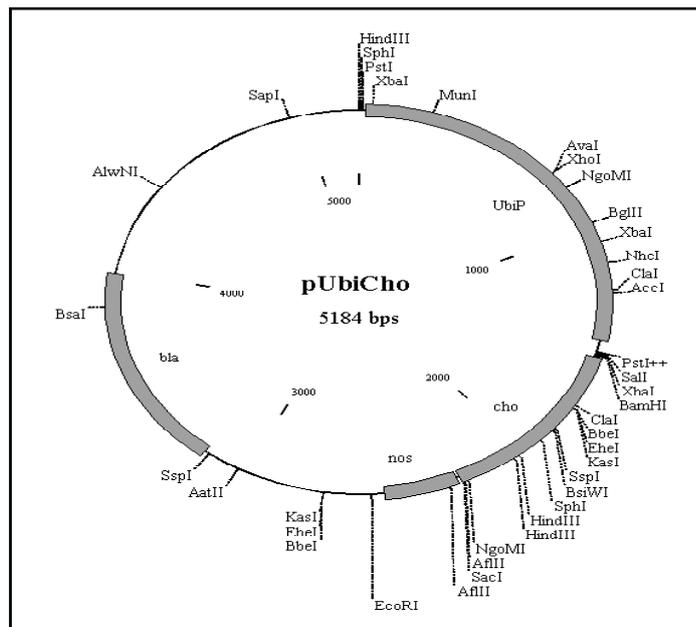


Figure: 3.3 Construct card for pUbi-Harcho.

Figures 3.2 and 3.3 show the detailed description of pUbi-HarChit and pUbi-HarCho constructs respectively

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3.1.1.2 Transformation with *pVst-HarChit* and *pVst-HarCho*:

IZEs were transformed with *pVst-HarChit* and *pVst-HarCho* using protocols described previously. A total of 31 plants were selected using selection agent *BASTA*.

3.1.1.3 Molecular Verification of *pUbi-HarChit* and *pUbi-HarCho* transgenic plants:

Transgenic plants were selected on the basis of selection marker gene *Pat* which confers resistance against herbicide *BASTA*. To prove them further for the genes of interest verifications were done at molecular level.

The plants which were resistant to *BASTA* the selection agent were checked for the presence of *pUbi-HarChit* and/or *pUbi-HarCho* by Southern Blot analysis. Details of the procedure can be seen in the section 2.2.5.5. Southern Blot analysis not only confirmed the presence of the gene constructs but also gave us the information about integration pattern and number of gene constructs integrated in the genome.

DNA was isolated from all the *BASTA* resistant plants and was run on 0.8% agarose gel by electrophoresis along with negative (wheat DNA from Florida genotype) and positive controls (Plasmid DNA). DNA from each plant was divided into three portions. One portion was undigested; the other portion was digested with an endonuclease that cuts once in the gene construct to make it linearized and the third portion was digested with endo-nuclease /endo-nucleases which cut the expression cassette (promoter, gene of interest and terminator) out of the construct. 25µg of each portion was run per lane. The lane containing undigested DNA was to confirm if there was any contamination of the Plasmid DNA. The lane containing DNA digested with endo-nuclease which linearizes Plasmid DNA was to compare and prove the hybridization procedure with positive control Plasmid DNA. The lane containing the DNA digested with endo-nucleases which cut the expression cassette out of Plasmid DNA was to compare the expression cassette in transgenic plant and Plasmid of gene construct. The lanes containing plasmid DNA were loading with 25pg of plasmid and additional 1 µg Herring's sperm DNA in order to keep the velocity of the plasmid DNA at par with the genomic DNA. Five plants along with positive and negative controls were run per gel. The DNA from the gel was then transferred to Nylon membranes by capillary transfer using protocols stated by Sambrook, 1989.

RESULTS

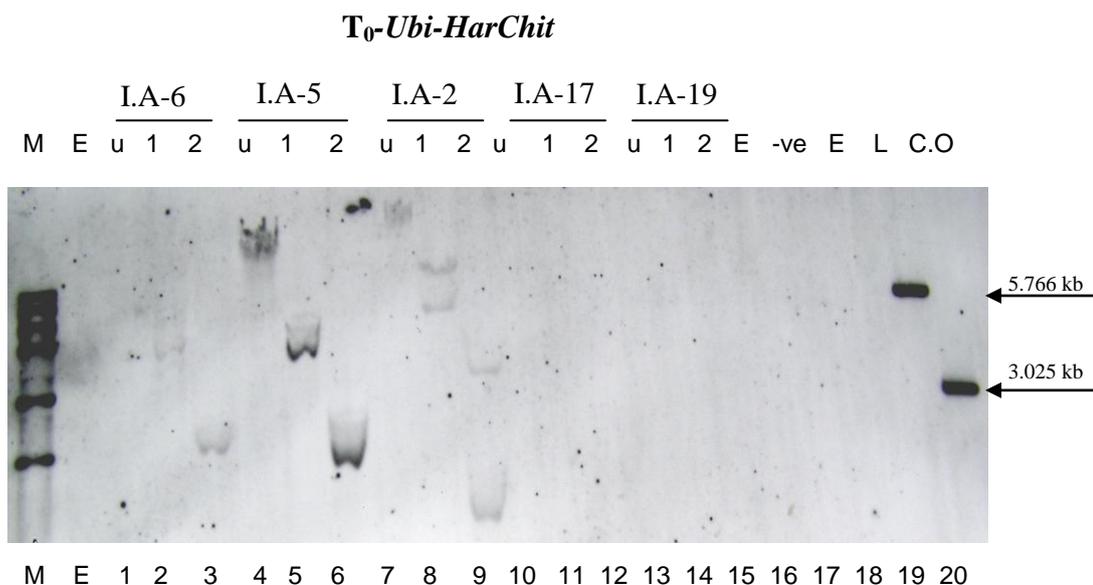


Figure: 3.6 Southern Blot analysis of T₀

In the above example, Plants I.A-5 and I.A-6 seems to have same integration pattern with single copy integration, Plant I.A-2 showed double copy integration with different sizes of positive fragments. I.A-17 and I.A-19 seem negative for *pUbi-HarChit*. Some times the expected fragments seem lighter than the fragments in the control. This size difference was due to difference in speed of genomic and plasmid DNA or some other reason will be seen after gene expression studies. The size of linear plasmid is 5.766 kb and gene expression cassette is 3.025 kb.

M =marker, **E** =empty lane, **U** =uncut, **1** = g-DNA digested with *EcoRI*, **2** =Digested with *Hind-III* and *EcoRI*, **-ve** = Non transgenic Florida plant DNA digested with enzyme used to cut the cassette from the plasmid. **L** =Plasmid DNA linearised by digestion with enzyme cutting only once in the plasmid *i.e EcoRI*. **C.O** =Plasmid DNA run after digestion with enzymes cutting on both ends of gene expression cassette (Promoter, gene of interest and terminator) in Plasmid gene construct. The first lane of every plant contains undigested genomic DNA, second lane is the DNA digested with enzyme that linearises the gene construct and the third lane contains DNA digested with enzymes which cut in gene construct on both sides of cassette. *EcoRI* was used to linearise the plasmid while *EcoRI* and *HindIII* to separate cassette. **1-3** = I..A-6, **4-6** = I.A-5, **7-9** = I.A-2, **10-12** = I.A-17, **13-15** = I.A-18. All the three probes of each putative transgenic plants (@ 25µg DNA per probe) were run on the 0.8% agarose gel along with positive (Plasmid DNA @ 25 pg per probe) and negative probes. DNA was transferred from this gel to the nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarChit* and the detection was made with CSPD substrate.

A 400 bp long fragment from *HarChit* gene and another 400 bp long fragment from *HarCho* gene were amplified from relevant gene constructs by using DIG-dNTPs to get DIG labelled fragments. These DIG-labelled fragments were used for the detection hybridization using 20-25 ng/mL of hybridization solution. Chemiluminescence's detection was done with CSPD® and the images were developed on X-rays film.

The examples of the Southern Blot analysis with *pUbi-HarChit* and *pUbi-HarCho* can be seen in Figure 3.6 and 3.7 respectively. A total of seven plants had *pUbi-Harchit* construct. Out of

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these seven plants three had single copy of *pUbi-Harchit*, three had double copy integration and one had three to four copies integrated in the genome.

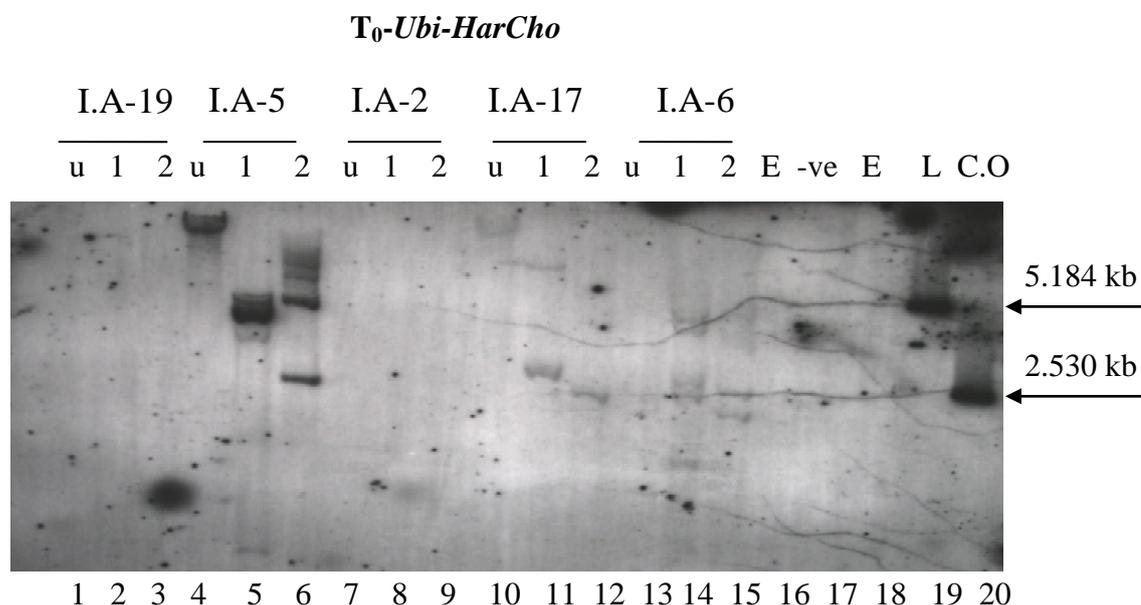


Figure: 3.7 Southern Blot analysis of T₀.

The above example shows single copy (I.A-17), double copy (I.A-5) and five copy (I.A-6) integration. In positive plants for *pUbi-HarCho* all 1s should at-least have one band equal to a band in L and one band equal to a band in C.O.

U= undigested g-DNA, 1= g-DNA digested with *EcoRI*, 2= g-DNA digested with *Pvu-II*. Lanes 1-3=I.A-19, Lanes 4-6= I.A-5, Lanes 7-9= I.A-2, Lanes 10-12= I.A-17, Lanes 13-15= I.A-6, Lanes 16 and 18 are empty while 17 contains -ve control. Lane 19= Plasmid DNA digested with *EcoRI* for linearization of Plasmid. Lane20 contains Plasmid DNA digested with *Pvu-II* to cut the expression cassette out of it. Digested and undigested DNA of all the five putative plants (@ 25 µg per probe) along with positive (Plasmid DNA was run @ 1 µg) and negative controls was run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarCho* and the detection was made with CSPD substrate.

Seven plants (*I.A-1, I.A-3, I.A-4, I.A-4, I.A-5, I.A-6, I.A-17* and *I.A-18*) were positive for *pUbi-Harcho* and out of these seven plants six were already positive for *pUbi-Harchit*. *I.A-2* was positive for *pUbi-Harchit* but proved negative for *pUbi-Harcho*. Similarly *I.A-17* was negative for *pUbi-Harchit* but proved positive for *pUbi-Harcho*. Out of the seven plants positive for the *pUbi-Harcho* three showed single copy integration, one showed two copies, others showed four, five and seven copy integration.

In summery six plants showed the co-integration of both *pUbi-Harchit* and *pUbi-Harcho* constructs while single construct (once *pUbi-Harchit* and once *pUbi-Harcho*) was present in couple of plants. The rest of the plants were either having only *bar* gene or were wrongly selected. In the above showed example plant *I.A-19* is shown as a plant that is resistant to

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BASTA herbicide and supposedly have *pat* gene but does not contain both the genes of interest.

3.1.1.4 Molecular Verification of *pVst-HarChit* and *pVst-HarCho* transgenic plants:

Putative transgenic plants selected through selection agent *BASTA* were further verified at molecular level for the presence of *pVst-HarChit* and *pVst-HarCho* gene constructs. DNA was isolated from all of the 31 *BASTA* resistant plants and Southern Blot analysis was done with the DIG labelled fragments of 400 bp for both *HarChit* and *HarCho* gene specific probes. DNA of all the plants was run on the agarose gel by electrophoresis in batches of three lanes. The first lane contained undigested DNA of the transgenic plant, second lane contained DNA digested with endo-nuclease that cuts the plasmid once to linearize and the third lane contains DNA digested with endo-nuclease that cuts the gene expression cassette out of the plasmid construct. For *pVst-HarChit* and *pVst-HarCho* both, *Kpn-I* was used for linearization and *Sfi-I* for cassette out. Each gel contained positive and negative controls as well. The DNA was transferred to the nylon membranes and hybridized separately with 400 bp long single stranded fragment amplified from *HarChit* and *HarCho* genes and labelled with DIG. Chemiluminescence's detection of DIG was done with CSPD® substrate. The results of Southern Blot analysis showed that out of 31 plants only five were positive for both *pVst-HarChit* and *pVst-HarCho* while one plant was having only *pVst-HarCho*. The rest of the plants were either having *pat* gene or were false selection. Only one plant had single copy integration for *pVst-HarChit*, the rest of the plants were having multicopy integrations for both the gene constructs. The copy number of integration varied from 1-5 for *pVst-HarChit* and 2-7 for *pVst-HarCho*. In the plant "I.A-8" one band in the lane reserved for cassette out showed heavier band than the expected while the bands in the lane reserved for linearized plasmid were normal. The rest of the plants showed at least one band equal to the band in the positive control lanes. The figure below shows the Southern Blot analysis for *pVst-HarCho* construct of all the positive plants.

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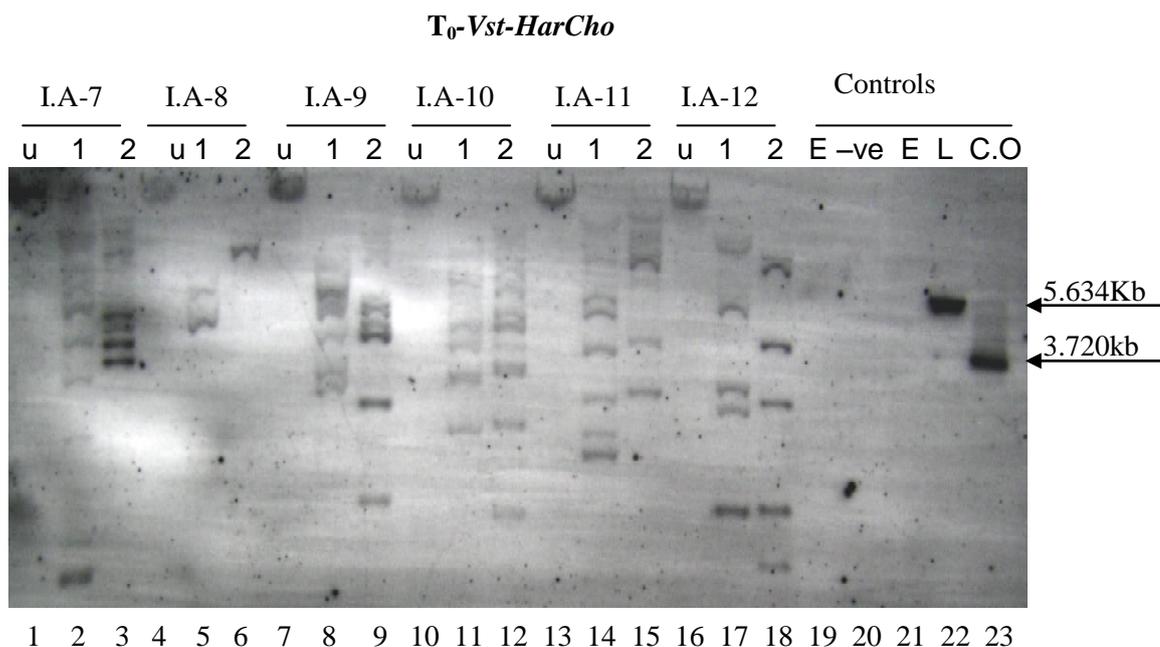


Figure: 3.8 Southern Blot analysis of T₀

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarCho* and the detection was made with CSPD substrate.

Southern Blot analysis of the putative transgenic plants showed that 6 plants are positive for *pVst-HarCho*. Five out of them were also positive for *pVst-HarChit* while I.A-8 was negative for *pVst-HarChit*. They showed high copy number in general except for Plant2 who had 1-2 copies.

U= Lane undigested g-DNA, 1= Lane with g-DNA digested with endo-nuclease that linearizes the gene construct. In this case it is *Kpn-I*, 2 = g-DNA digested with endonuclease that cuts the cassette out of the gene construct. In this case it is *Sfi-I*. Lanes 1-3 = I.A-7, Lanes 4-6 = I.A-8, Lanes 7-9 = I.A-9, Lanes 10-12 = I.A-10, Lanes 13-15 = I.A-11, Lanes 16-18 = I.A-12, Lanes 19-23 = controls and empty lanes in between.

In summery a total of five plants with co-integration of *HarChit* and *HarCho* under stress inducible promoter were found. One plant had only *HarCho* gene under stress inducible promoter in its genome.

3.1.1.5 Segregation in the Transgenic Generations:

Segregation analysis was done based on the *BASTA* resistance in the coming generations of the primary transformants. Hundred seeds were sown per tested line and sprayed with *BASTA* herbicide 10 days and 17 days after germination with 150 mg/L and 200 mg/L respectively. The surviving plants were counted to calculate the segregation ratios in the transgenic lines. As shown below in the table some lines showed single locus integration for *pat* gene and 3:1 ratio was achieved. In other lines this ratio varied greatly for example progeny of I.A-10

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completely died on *BASTA* herbicide spray. In line I.A-18 this ratio looked reversed of the single locus integration *i.e* 1:3 instead of 3:1. In other lines like I.A-4 and I.A-12 it looked perfectly single locus integration. The matter was not studied further in detail because it was beyond the scope of this project. The plants were advanced further for molecular analysis of genes of interest and phyto-pathological studies.

Table: 3.2 Segregation analysis for the over expression Transgenics

Transgenic line	Percent germination	No. of Dead Plants	No. of surviving Plants	Percentage of surviving Plants
Over expression lines with constitutive promoter:				
I.A-3	97	20	77	79.38
I.A-4	98	21	77	78.58
I.A-5	100	35	65	65.00
I.A-6	96	10	86	98.58
I.A-18	100	74	26	26.00
Over expression lines with inducible promoter:				
I.A-7	98	82	16	16.33
I.A-8	95	19	76	80
I.A-9	100	76	24	24
I.A-10	92	92	-	-
I.A-11	100	70	30	30
I.A-12	100	21	79	79

3.1.1.6 Molecular Verification of transgenes in Segregating lines:

Self pollinated T₀ plants were harvested after they reached the maturity. The seeds were stored at 4°C for eight weeks to break seed dormancy. When the seed dormancy period was over some seeds were taken and were sown in the green house under recommended conditions (18°C /14°C day/night, 16 h light of 23000-25000lux). In some cases embryo rescue was done to save time (to be used in breaking seed dormancy) and plantlets were taken on ½ MS from immature embryos of T₀ spikes as reported by Leckband, 1997. T₁ seedlings were sprayed twice with 150 mg/L and 200 mg/L *BASTA* solution 10 days and 17 days after germination to select the transgenic plants and rouge out the segregating non transgenics.

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3.1.1.7 Verification of First Transgenic generation (T₁) for *pUbi-HarChit* and *pUbi-HarCho*:

DNA was isolated from the *BASTA* resistant T₁ plants and digested with *Hind-III* and *EcoRI* for *pUbi-HarChit* and *Pvu-II* for *pUbi-HarCho*, the endo-nuclease/endo-nucleases which cut the cassettes out of the plasmids. This DNA along with positive and negative controls was loaded and run onto 0.8% agarose gel by electrophoresis. Each lane contained 25 µg of genomic DNA while 25 Pico grams were loaded for positive control plasmid. DNA was transferred to onto the nylon membrane by capillary transfer and hybridized with DIG labelled 400 bps long single stranded DNA fragment amplified separately from *HarChit* and *HarCho* genes of the transformation constructs and detected with CSPD® substrate. The detailed procedure is explained in section 2.2.5.5.

T₁-Ubi-HarChit

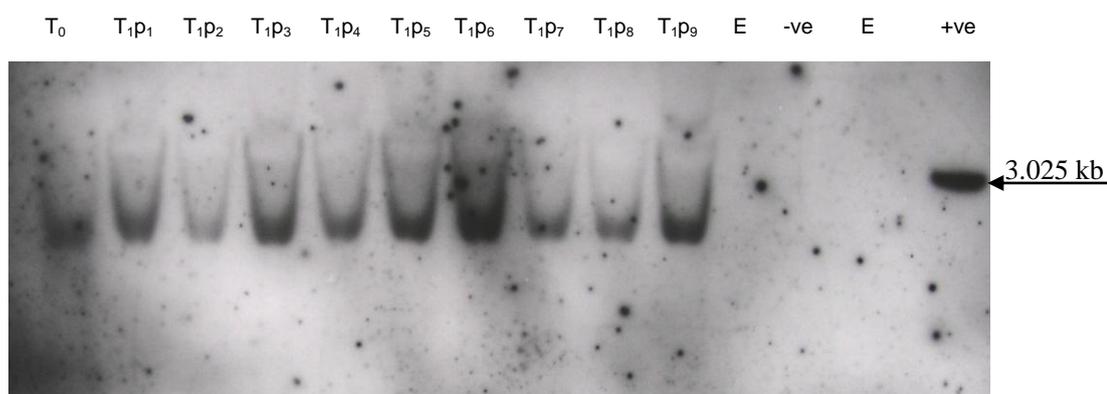


Figure: 3.9 Southern Blot analysis of T₁.

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarChit* and the detection was made with CSPD substrate.

T₀ is the lane representing primary transformant and the next 9 lanes represent plants selected after selection pressure was put to the progeny of primary transformant “I.A-5”. All the lanes show only one band of same size. Empty lanes as well as lanes with DNA from non transgenic plants showed no band. DNA run in all the lanes was digested with the same endonuclease that cuts the cassette out of *pUbi-HarChit* construct. Digested Plasmid DNA along with herring’s sperm DNA was run in the last lane as positive control.

An example of the detection for *pUbi-HarChit* by Southern Blot analysis is shown in figure 3.9. As is shown here all the *BASTA* resistant plants were also having the gene of interest and the integration pattern was also the same as it was in the T₀ generation. The negative control and empty lanes did not have any positive bands to prove the reliability of the results. The

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same kind of the results were produced for all the lines produced. Southern Blot analysis showed the same results for *pUbi-HarCho*.

Segregating behaviour of transgenic to non transgenic is stated separately in table 3.2. The results showed that it was possible to successfully advance the transgenic lines to next generation. The integration pattern was also conserved in all lines as is shown in figure 3.9 Figure 3.9 shows Southern Blot analysis for *pUbi-Harchit* in the progeny of transgenic plant “I.A-5” which showed single copy integration in T₀ also showed the same in all the plants tested in T₁ generation.

3.1.1.8 Molecular Verification of *pVst-HarChit* and *pVst-HarCho* in the transgenic Generations:

Transgenic seeds of the T₀ plants were sown and the plants were selected by spraying *BASTA* herbicide twice 10 days after germination at an interval of one week as is stated in the section 2.2.4.5. The selected plants were then tested for the presence of *pVst-HarChit* and *pVst-HarCho*.

The DNA was isolated from the selected plants and digested with *Sfi-I* endo-nuclease that cuts gene expression cassette out of both the *pVst-HarChit* and *pVst-HarCho* plasmids. DNA of 8-10 plant from each line was run separately on two different gels at the rate of 25 µg per lane. Each lane represented a different plant. Negative control DNA was also treated alike. Positive plasmid DNA digested with *Sfi-I* was also run at 25 Pico grams plus 1 µg herring's sperm DNA. The DNA was transferred to the nylon membrane and Southern Blot analysis was done using DIG labelled 400 bp long single stranded *HarChit* and *HarCho* based probes following the protocols described in the section 2.2.5.5.

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T₁-Vst-HarChit

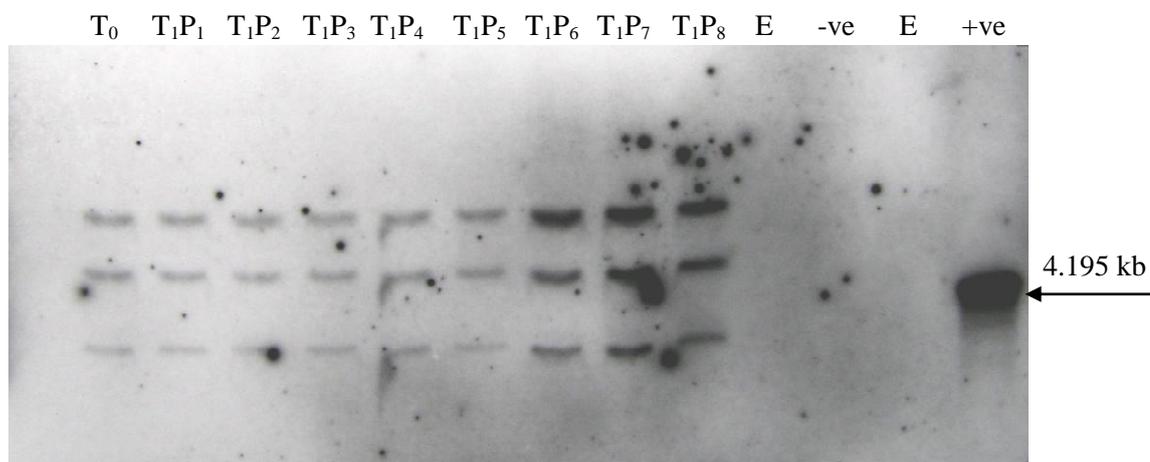


Figure: 3. 10 Southern Blot analysis of T₁ line I.A-11 for Inducible promoter construct with chitinase gene specific probe

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarCho* and the detection was made with CSPD substrate.

Southern Blot analysis of the T₁ selected plants showed that the integration of *pVst-HarChit* in the next generation is the same as it was in the primary transformants as is shown here (see figure 3.8 and plant *I.A-11* and lane C.O to compare with this picture). T₀ plant and the T₁ selected plants looked the same with a copy number of three proving the successful transfer of *pVst-HarChit*.

The results of the Southern Blot analysis showed that it was possible to advance the transgenics with *pVst-HarChit* and *pVst-HarCho* to the next generation. Even the line “I.A-10” which showed the death of all the T₁ plants after *BASTA* sprays showed the advancement of *pVst-HarChit* and *pVst-HarCho* to T₁ generation. Figure 3.10 shows Southern Blot analysis of line “I.A-11” with *pVst-HarChit*. T₁ generation seems to be exactly the same as is it in the T₀ generation. The same were the results for all other lines for *pVst-HarChit* as well as for *pVst-HarCho*.

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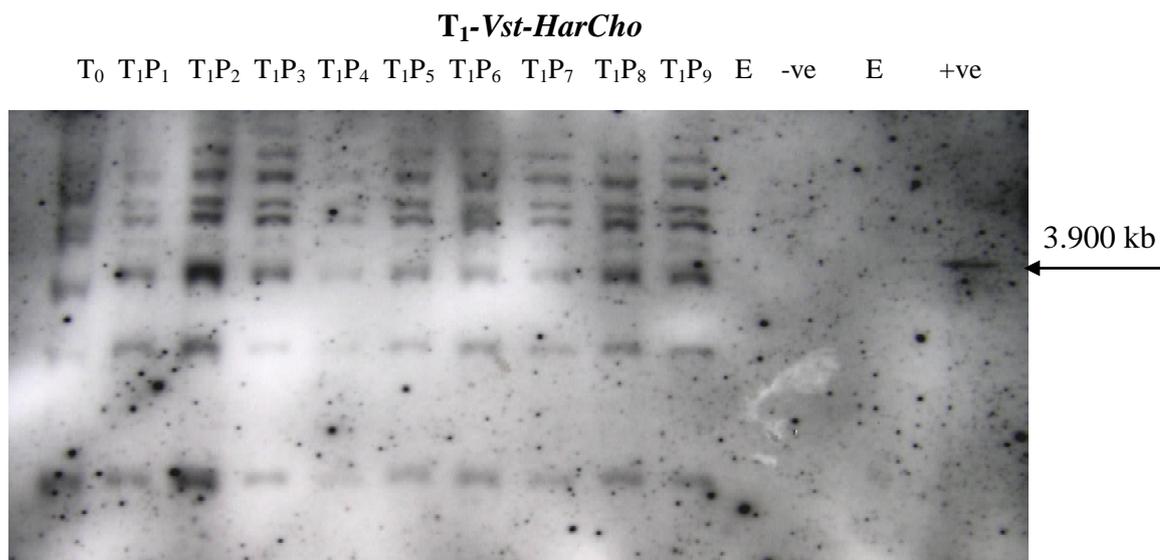


Figure: 3.11 Southern Blot analysis of T₁ line IA-10 for Inducible promoter construct with *HarCho* gene specific probe

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarCho* and the detection was made with CSPD substrate.

As is shown here *pVst-HarCho* was successfully transferred to the next generation. T₀ and the selected plants in the T₁ generation showed exactly the same integration pattern. T₀ is DNA lane from primary transformant IA-10 and the next 9 lanes contain *Sfi-I* digested DNA from 9 independent plants of T₁ progeny of this plant. E= empty lanes and -ve= negative control (non transgenic Florida DNA. +ve= plasmid DNA.

Figure 3.11 shows the Southern Blot analysis for *pVst-HarCho* in line “IA-10”. The T₀ plant for this line contained the seven bands and same bands are present in all the T₁ generation plants. And one of these bands is corresponding exactly to the band in positive control. The negative control and the empty lanes show no bands proving the authenticity of the experiments.

In Summery, it is proved that it is possible to successfully advance the transgenic lines having *HarChit* and *HarCho* genes under stress inducible promoter to the next generation.

3.1.1.9 Second transgenic generation T₂:

Embryo rescue was done from the immature embryos of the T₁ generation plants to get plants for the next generation T₂. All the plants tested in T₂ showed the same behaviour as was seen in T₀ and T₁ generation. Figures 3.12 and 3.13 show Southern Blot analysis for *pUbi-Harchit* and *pUbi-Harcho* detection in T₂ generations of lines named “IA-6” and “IA-18” respectively. For the Southern Blot analysis of T₂ generation, DNA was isolated from the T₂

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plants and 18 plants were checked in the T₂ generation for the presence of *pUbiHarChit* and *pUbiHarCho*. DIG labelled probes of around 400 bp were prepared from the concerned genes. DNA was digested with *Hind-III* and *EcoRI* for *pUbi-HarChit* Southern Blot analysis and *Pvu-II* for *pUbi-HarCho* analysis. Positive and negative probes were also loaded on the gels and the process was completed according to protocols given in the section 2.2.5.5.

T₂-ubi-chit

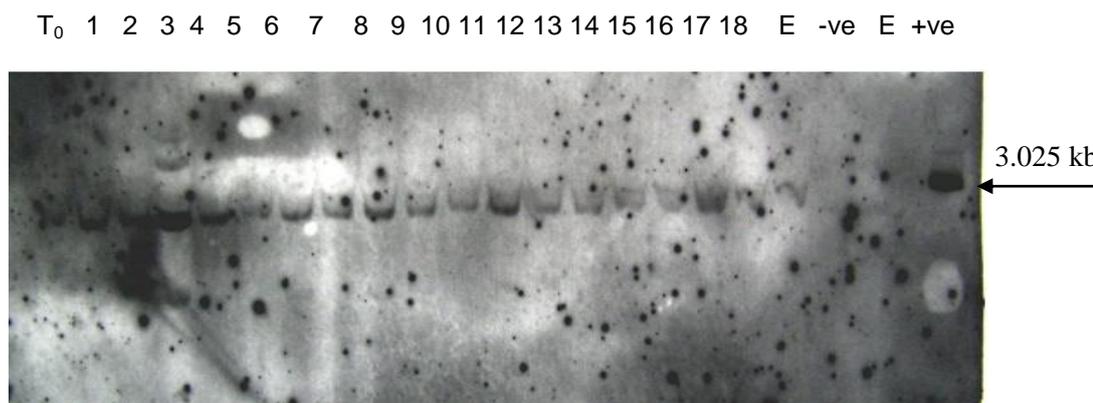


Figure: 3.12 Southern Blot analysis of T₂ line IA-6 *HarChit* gene specific probe.

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarChit* and the detection was made with CSPD substrate.

Same integration pattern is shown by all the tested *BASTA* positive plants in the T₂ generation of 'IA-6' for *pUbi-HarChit* as is shown by primary transformant (See figures 3.7 and 3.9 for comparison). T₀ is primary transformant and lanes 1-18 contain 18 *BASTA* positive plants in the T₂ generation. T₀= primary transformant, 1-18= *BASTA* selected plants from the progeny of T₁ plant. E= empty lanes, +ve = positive control and -ve = negative control. DNA from all the probes was digested with *Hind-III* and *EcoRI* endonucleases and a 3.025 band was expected on all the positive plants of progeny and plasmid control while no band was expected in the negative control and empty lanes.

Figure 3.12 shows the progeny of transgenic plant named "IA-6" which was tested with *HarChit* specific probes. All the 18 plants presented showed the same behaviour as was shown by T₀ plant. Positive and negative controls showed expected results with no bands in negative controls and expected bands on positive control.

The figure 3.13 shows an example of the T₂ plants of the line 'IA-18' with *pUbi-HarCho* probe. It is evident from the figure that all the plants in the T₂ generation showed exactly the same integration pattern as was in the T₀ plant and T₁ generation. One dark band was seen in all the plants and as well as in the positive control.

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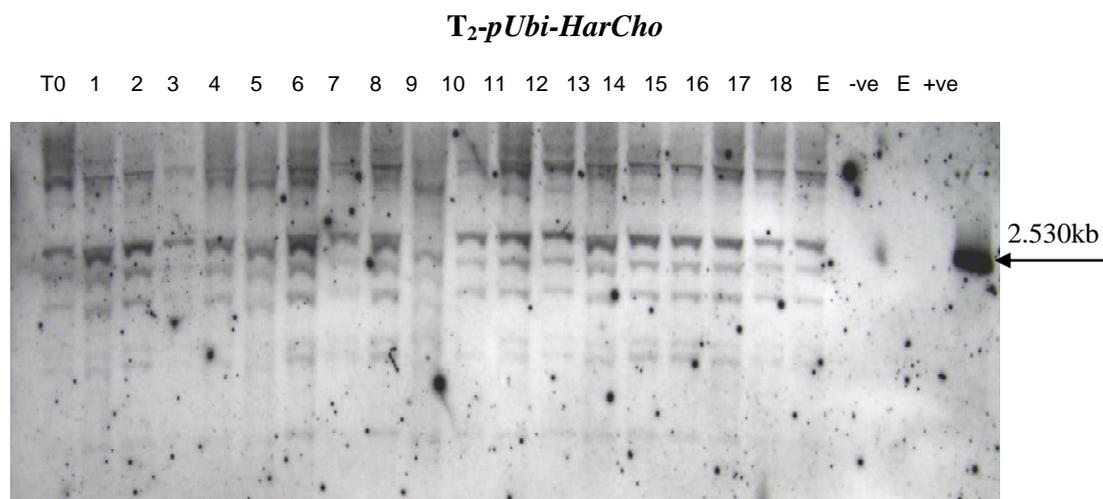


Figure: 3.13 Southern Blot analysis of T₂ line I.A-18 with *Har-chitosanase* gene specific probe.

DNA of all the probes along with positive and negative probes were run on 0.8% agarose gel and transferred to nylon membrane by capillary method and cross linked later. DNA on this membrane was hybridized to the DIG labelled 400 bp single stranded DNA probe from *HarCho* and the detection was made with CSPD substrate.

T₀ = primary transformants, **1-18**= eighteen plants from first generation of line 'I.A-18', E= empty lane, +ve= plasmid digested with *Pvu-II*. Same integration pattern is shown by all the tested plants of T₂ generation for *pUbi-HarCho* as is shown by primary transformant. For comparison, see figures 3.6 and 3.9.

In summery, it was possible to advance transformed *HarChit* and *HarCho* genes under constitutive and inducible promoters to the next generation.

3.1.1.10 Expression analysis of *pUbi-HarChit* and *pUbi-HarCho* transgenes by Northern Blot:

All the plants which were proved to have *pUbi-Harchit* and / or *pUbi-Harcho* by Southern Blot analysis were checked for the expression of these genes in primary transformants as well as in T₁ generation. RNA was isolated from the leaves of the transgenic and non transgenic control plants and run on the degrading agarose gel at the rate of 15µg per lane per plant. Five lanes were reserved for each transgenic plant, one lane for T₀ RNA and four for T₁ only one lane was reserved for non transgenic control. RNA was transferred to the positively charged Nylon membrane.

3.1.1.11 Northern Blot analysis of Transgenics and their progenies for *Har-Chit* gene expression from *pUbiHarchit* construct:

Out of the seven Southern Blot analysis positive plants for *pUbiHarchit* six plants showed the expression in T₀ and T₁ generation and only one plant did not show the expression of this gene in T₀ and T₁ plants. Some detailed results are shown below in the example presented in

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figure 3.14. Figure 3.14a shows the integrity of the RNA used on all the probes for Northern Blot analysis in the example.

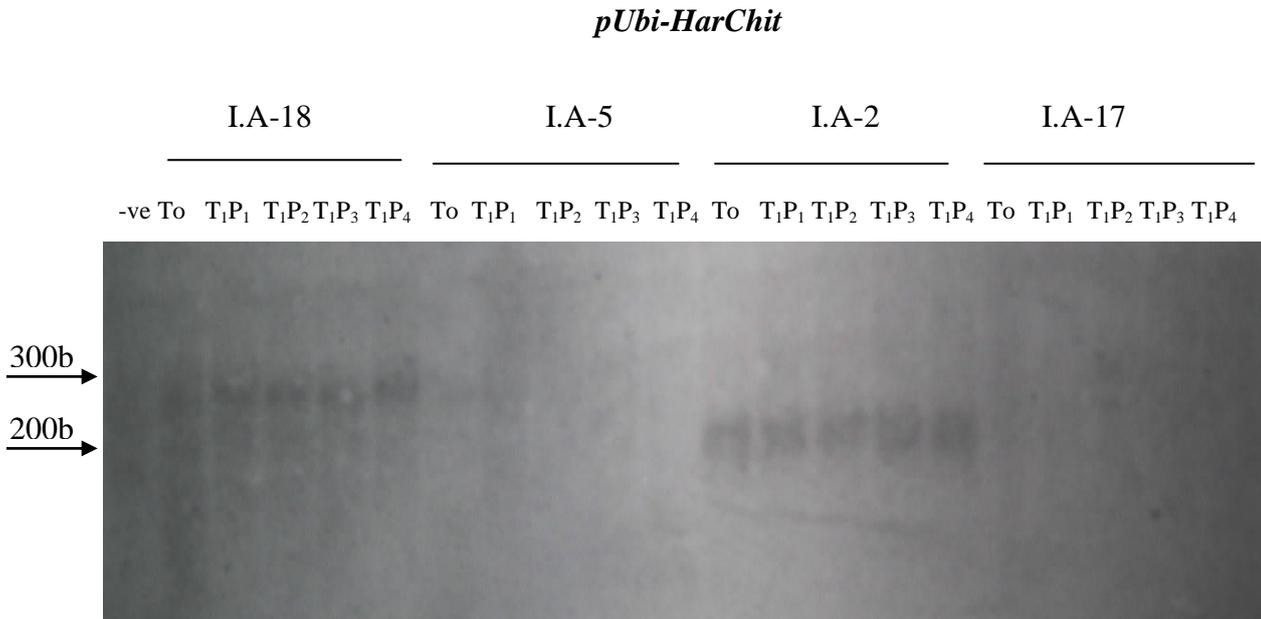


Figure: 3.14a Northern Blot analysis of T₀ and T₁ generation with *pUbi-HarChit* construct.

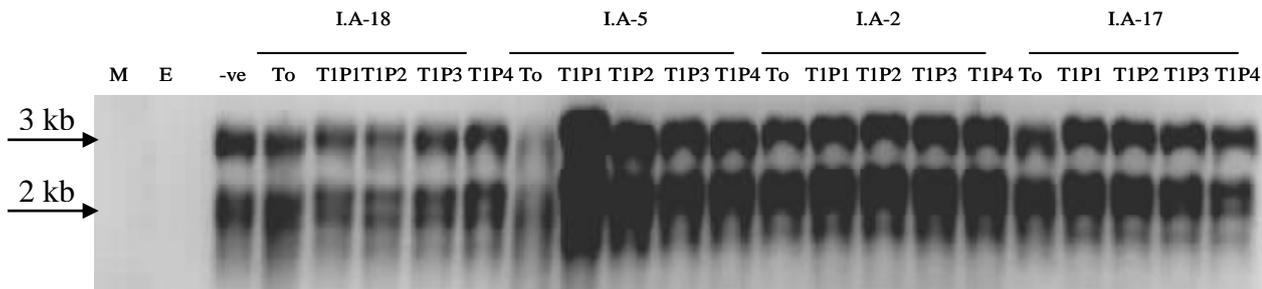


Figure: 3.14b. Control gel for Northern of T₀ and T₁ generation with *pUbi-HarChit* construct.

Freshly isolated RNA from young spikes of the entire Southern Blot analysis positive T₀ and selected T₁ progeny plants was run on 1% denaturation gel at the rate of 15 µg per probe/lane and transferred to the nylon membrane by capillary method under clean environment and cross linked later. This RNA was then hybridized with 400 bp long radio actively labelled *HarChit* specific single stranded DNA.

Figures: 3.14a and b one exemplary gel is presented with I.A-18, I.A-5, I.A-2 and I.A-17 transgenic lines. -ve = is the RNA from control non transgenic plant. T₀ is the RNA from primary transgenic plant and T₁P₁ to T₁P₄ is the RNA from four selected plants from progeny of the primary transformants.

The results of the Northern Blot analysis showed that six out of seven Southern Blot analysis plants were showing the expression of the *HarChit* while I.A-5 which was positive for *pUbi-HarChit* did not show any expression of it. I.A-17 was proved negative for *pUbi-HarChit* was again negative for Northern blot analysis. The results are shown in the example presented in figure 3.14.

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3.1.1.12 Northern Blot analysis of Transgenics and their progenies for *Har-Cho* gene expression from *pUbi-Harcho* construct:

Out of seven plants proved to contain *pUbi-Harcho* only two (I.A-1 and I.A-5) did not show any expression for the *HarCho* gene while the rest showed the expression. I.A-5 did not even show the expression for *Harchit* while I.A-1 showed the expression for *HarChit* only although both the gene constructs were present in these plants and their progenies. An example is presented here with control gel picture showing the integrity of the RNA in all the probes.

pUbi-HarCho

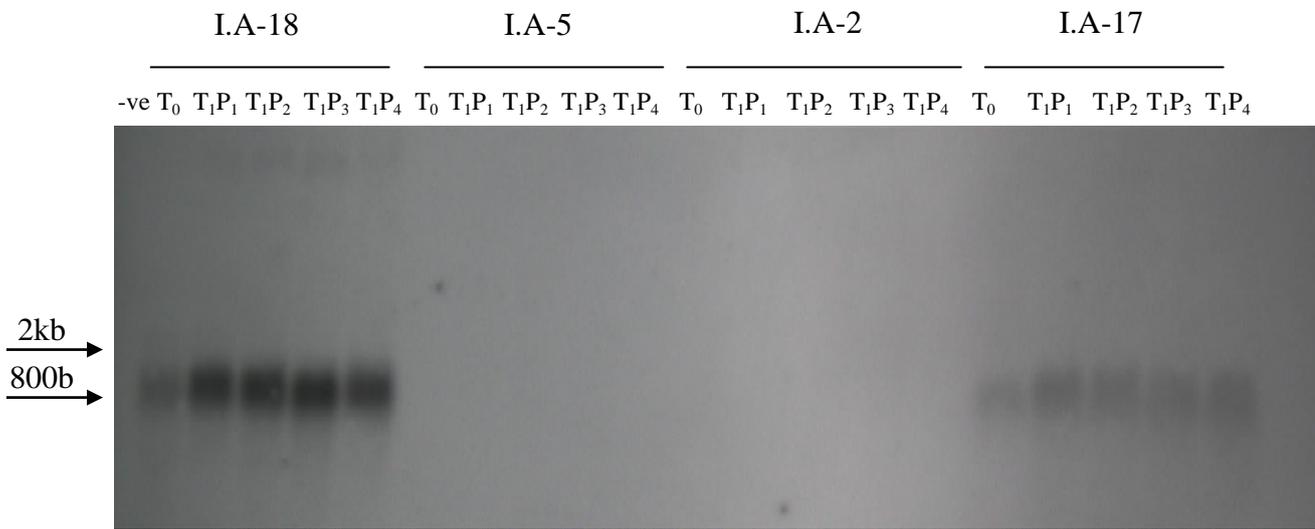


Figure: 3.15a Northern Blot analysis of T₀ and T₁ generation with *pUbi-HarCho* construct.

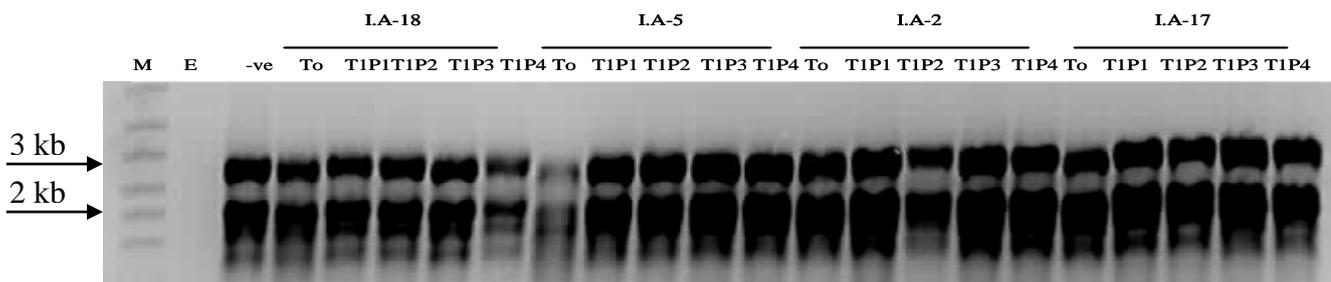


Figure3.15a. control gel for Northern Bl of T₀ and T₁ generation with *pUbi-HarCho* construct.

Freshly isolated RNA from young spikes of the entire Southern Blot analysis positive T₀ and selected progeny plants was run on 1% degeneration gel at the rate of 15 µg per probe/lane and transferred to the nylon membrane by capillary method under clean environment and cross linked later. This RNA was then hybridized with 400 bp long radio actively labelled *HarCho* specific single stranded DNA.

-ve = non transgenic plant RNA, T₀ = RNA from primary transgenic plants, T₁P₁- T₁P₄ = four selected Plants from the progenies of tested plants. Figure 3.15a shows the integrity of the RNA used for Northern in all the probes.

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Figure: 3.15a shows the expression of *HarCho* gene in T₀ and T₁ plants of plants “I.A-18” and “I.A-17”. “-ve” control, and “I.A-2” were expected to show no expression because “-ve” was non transgenic control plant and “I.A-2” was proved negative by Southern Blot analysis.

Figure 3.15 shows the expression of *HarCho*. I.A-18 and I.A-17 are expressing the gene in T₀ as well as in T₁ generation while there is no expression in I.A-2 and I.A-5. I.A-2 did not show the integration of *pUbi-HarCho* during Southern Blot analysis but this construct was integrated in I.A-5.

The conclusion of molecular analysis of *pUbi-Harchit* and *pUbi-Harcho* integration and expression is that six transgenic plants were recovered with the co-integration of *pUbi-HarChit* and *pUbi-HarCho* genes into wheat genome. One plant had only *pUbi-HarChit* and one had only *pUbi-HarCho*. The successful transfer of transgenes was also proved to the progeny of primary transformants.

The expression of transgenes was not possible in all the transgenic plants and progeny. One plant that had got both the genes and marker gene *pat* showed only the expression of marker gene but did not show the expression of both the genes of interest. The other plant which contained both genes showed the expression of *HarChiy* only.

3.1.1.13 rt-PCR analysis for the expression of *pVst-HarChit* and *pVst-HarCho* constructs:

In order to check the expression of the *HarChit* and *HarCho* in the transgenics *Vst1* promoter was induced by wounding with sea sand on the leaf surfaces. RNA was isolated eight hours after induction as reported by Leckband, 1997. RNA was treated with DNAase and First strand c-DNA was synthesized from this RNA using oligo dt₁₈ primers and c-DNA synthesizing kit from Fermentas (St. Leon, Germany).

Induction of *HarChit*:

PCR reactions were done with the synthesized c-DNA using *HarChit* specific primers. A band of around 400bp was expected on the transgenic plants with induced *Vst1* promoter as well as on *pVst-HarChit* plasmid DNA. All the five plants which were Southern Blot analysis positive for *pVst-HarChit* were checked. The PCR product from all of them was compared with negative (non transgenic) control, PCR (H₂O was used as second negative to check any contamination) and positive control (plasmid DNA specific product). The PCR product was also sequenced to confirm the *HarChit* induction in transgenics.

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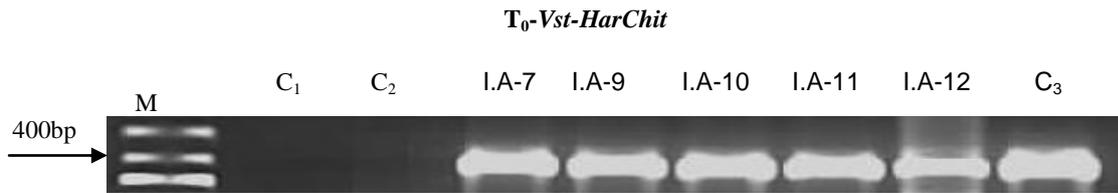


Figure: 3.16a. rt-PCR of the T₀pVst-HarChit positive plants.

Positive control showed the band of the expected size as did all the positive plants while the negative controls never showed the presence of any transcript from *HarChit*. PCR reaction showed the presence of *HarChit* transcript after *VstI* promoter induction with wounding stress.

M= Marker, C₁= reagents control with H₂O as template, C₂ = negative control with template from non transgenic plant. C₃ = positive control with template from *pVst-Chit* plasmid.

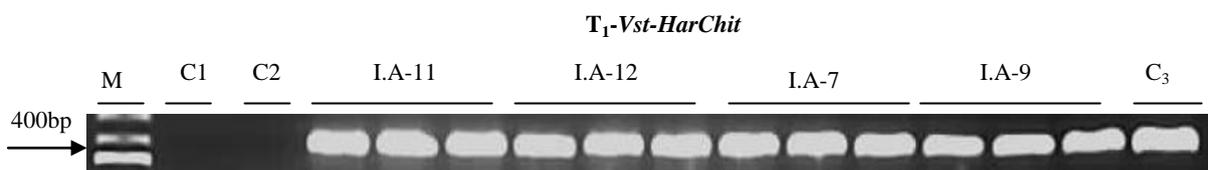


Figure: 3.16b rt-PCR of the T₁pVst-HarChit positive plants from T₀ progenies

Positive control showed the band of the expected size as did all the positive plants while the negative controls never showed the presence of any transcript from *HarChit*. PCR reaction showed the presence of *HarChit* transcript after *Vst* promoter induction with wounding stress in the progenies of *pVst-HarChit* positive plants.

M= Marker, C₁= reagents control with H₂O as template, C₂ = negative control with template from non transgenic plant. C₃ = positive control with template from *pVst-HarChit* plasmid.

Figures 3.16a and 3.16b shows the presence of *HarChit* transcription on wound induction in all the T₀ plants as well as in the T₁ plants. The positive and negative controls gave the expected results. The positive control with a template of plasmid DNA gave a band exactly like the transgenic plants gave. PCR negative control with H₂O as template did not give any band and same did when the negative control for transgenics where c-DNA from non transgenic plant was taken as template.

Induction of *HarCho*:

PCR reactions were done with the synthesized c-DNA using *HarCho* specific primers. A band of around 400bp was expected on the transgenic plants with induced *Vst* promoter as well as on *pVst-HarCho* plasmid DNA. PCR product from all the six *pVst-HarCho* Southern Blot analysis positive plants and non transgenic control was compared with negative H₂O template control.

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T₀-Vst-HarCho



Figure: 3.17a. rt-PCR of the *T₀pVst-HarCho* positive plants.

Positive control showed the band of the expected size as did all the positive plants while the negative controls never showed the presence of any transcript from *HarChit*. PCR reaction showed the presence of *HarCho* transcript after *Vst* promoter induction with wounding stress. The PCR products with I.A-7 and I.A-9 showed two bands which may be due to incomplete integration of a copy of gene construct.

M= Marker, C₁= reagents control with H₂O as template, C₂ = negative control with template from non transgenic plant. C₃ = positive control with template from *pVst-HarCho* plasmid.

T₁-Vst-HarCho

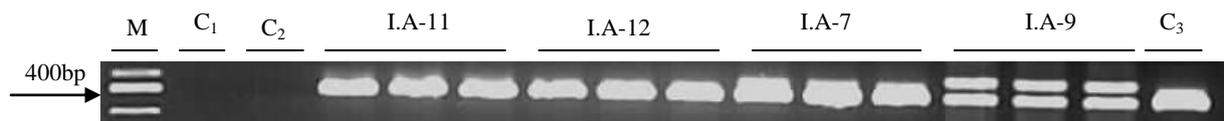


Figure: 3.17b. rt-PCR of the *T₁pVst-HarCho* positive plants from *T₀* progenies

Positive control showed the band of the expected size as did all the positive plants while the negative controls never showed the presence of any transcript from *HarChit*. PCR reaction showed the presence of *HarCho* transcript after *Vst* promoter induction with wounding stress in the progenies of *pVst-HarChit* positive plants. The trend of double bands continued even in the *T₁* generation as is shown in the example above.

M= Marker, C₁= reagents control with H₂O as template, C₂ = negative control with template from non transgenic plant. C₃ = positive control with template from *pVst-HarCho* plasmid.

Figures 3.17a and 3.17b showed the presence of *HarCho* transcription on wound induction in all the *T₀* plants as well as in the *T₁* plants. The positive and negative controls gave the expected results. The positive control with a template of plasmid DNA gave a band exactly like the transgenic plants gave. PCR negative control with H₂O as template did not give any band and same did the negative control for transgenics where c-DNA from non transgenic plant was taken as template. I.A-9 continued the trend of double band in the *T₁* and *T₂* generation. This can be due to additional incomplete integration of the gene cassette as these plants were having multicopy integration of *pVst-HarCho*.

In summery, it was possible to transform wheat with *pVst-HarChit* and *pVst-HarCho* and induce both the genes on wound induction. Out of the six transgenic plants five were having the co-integration and expression of *pVst-HarChit* and *pVst-HarCho* and one was having only *pVst-HarCho*.

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Table: 3.3 Summary of the Gene Integration and Expression among *HarChit* and *HarCho* Transgenics.

No.	Lines	Southern Blot analysis		Northern/rtPCR	
		<i>HarChit</i>	<i>HarCho</i>	<i>HarChit</i>	<i>HarCho</i>
Transgenic Plants with Constitutive Promoter					
1	I.A-1	+	+	-	+
2	I.A-2	+	-	+	-
3	I.A-3	+	+	+	+
4	I.A-4	+	+	+	+
5	I.A-5	+	+	-	-
6	I.A-6	+	+	+	+
7	I.A-17	-	+	-	+
8	I.A-18	+	+	+	+
Transgenic plants with stress inducible promoter					
9	I.A-7	+	+	+	+
10	I.A-8	-	+	-	+
11	I.A-9	+	+	+	+
12	I.A-10	+	+	+	+
13	I.A-11	+	+	+	+
14	I.A-12	+	+	+	+

Table 3.3 gives an overview of the *HarChit* and *HarCho* transformation and expression in wheat. In total 14 transgenic plants were found with the gene/genes of interest, eight under the control of constitutive promoter and six under stress and disease inducible promoter. One line showed the integration of both *HarChit* and *HarCho* under *Ubiquitin* promoter but there was no expression, another plant showed the integration of both *HarChit* and *HarCho* but expression was found only on *HarCho*. Four plants showed the co-integration and expression of *HarChit* and *HarCho* under *Ubiquitin* constitutive promoter. One line showed integration and expression of *HarChit* only and two lines showed the integration and expression of *HarCho* only.

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The case was not a lot different when disease and stress inducible promoter was used. A total of six plants were found and five of them showed co-integration and expression of both the genes. One line showed the integration and expression of *HarCho* only.

3.1.1.14 Phyto-pathological experiments:

Two pathogens named *Fusarium graminearum* and *Erysiphe graminis* f.sp. *tritici* were used to check the effect of *HarChit* and *HarCho* genes expressed constitutively and induced under pathogen stress for the enhancement of plant response against diseases.

3.1.1.14.1 Powdery Mildew (*Erysiphe graminis* f.sp *tritici*):

Powdery Mildew is common disease found in many plant species but the pathogen is different for each plant species. The pathogen used in these experiments causes disease only in wheat. *E. graminis* is a biotrophic fungus and can be maintained only on the plant material. Pathogen was maintained on highly susceptible wheat genotype “Kanzler”

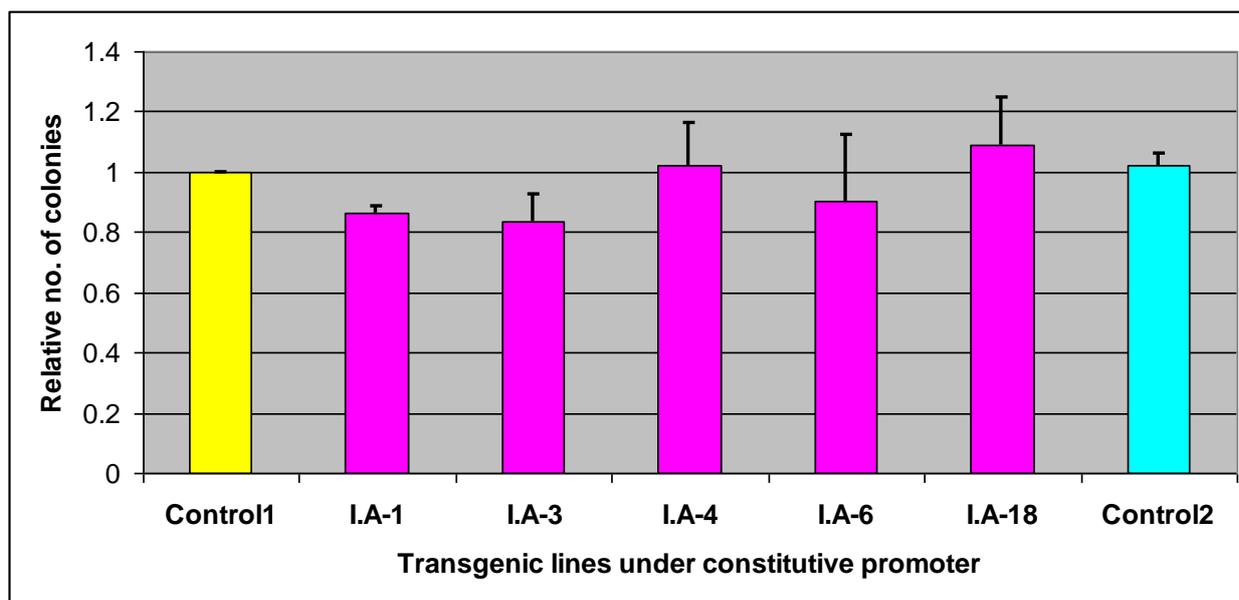


Figure: 3.18 Comparison of the Number of Colonies per leaf disk among Transgenics under Constitutive Promoter.

4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm². Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. First data were taken 9dpi for the number of developing colonies per leaf segment compared to the non transgenic control.

Figure: 3.18 shows the relative number of colonies developed on the leaf disks of the transgenic plants (having *HarChit* and/or *HarCho* expressed under constitutive promoter) compared to the non transgenic and transgenic control plants. Here

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control-1 is the non transgenic control while control-2 is the transgenic control with *gus* and *pat* constructs. The rest bars represent transgenic plants (I.A-1, I.A-3, I.A-3, I.A-4, I.A-6 and I.A-18).

The protocols used for inoculations of transgenic with PM for pathological analysis are stated in section 2.2.6.1. Leaf segments of 4 centimetres length were cut from plantlets at two leaves stage and cultured on the anti-senescence media containing benzimidazol and silver nitrate in 0.4% agar. Non transgenic control was compared with all the transgenic lines under study as well as with the transgenic control. All the inoculation plates were kept in the settling tower and powdery mildew inoculums were shot at them with a 500 psi pressure. It was made sure that 300-400 conidia should spread per cm² of the inoculation plate. The experiment was kept at 17°C overnight and then transferred to 18°C for the next three weeks. 16-8 hour light to dark conditions were maintained.

For leaf disk infection, nine days after inoculation the data were taken for the number of *Erysiphe* colonies growing on the inoculated leaf disks. The number of colonies was counted per leaf disk (4 centimetre long first leaf) in all the transgenic and control lines and then relative number of colonies was calculated by taking the number on control1 (Non transgenic control) as 1. The results shown above in Fig. 3.18 revealed a reduction of the number of colonies developed on all the transgenic lines except I.A-4. Control2 which is transgenic line containing only *BASTA* resistance gene and *gus* gene showed almost the same number of colonies as Control1 (Non transgenic control).

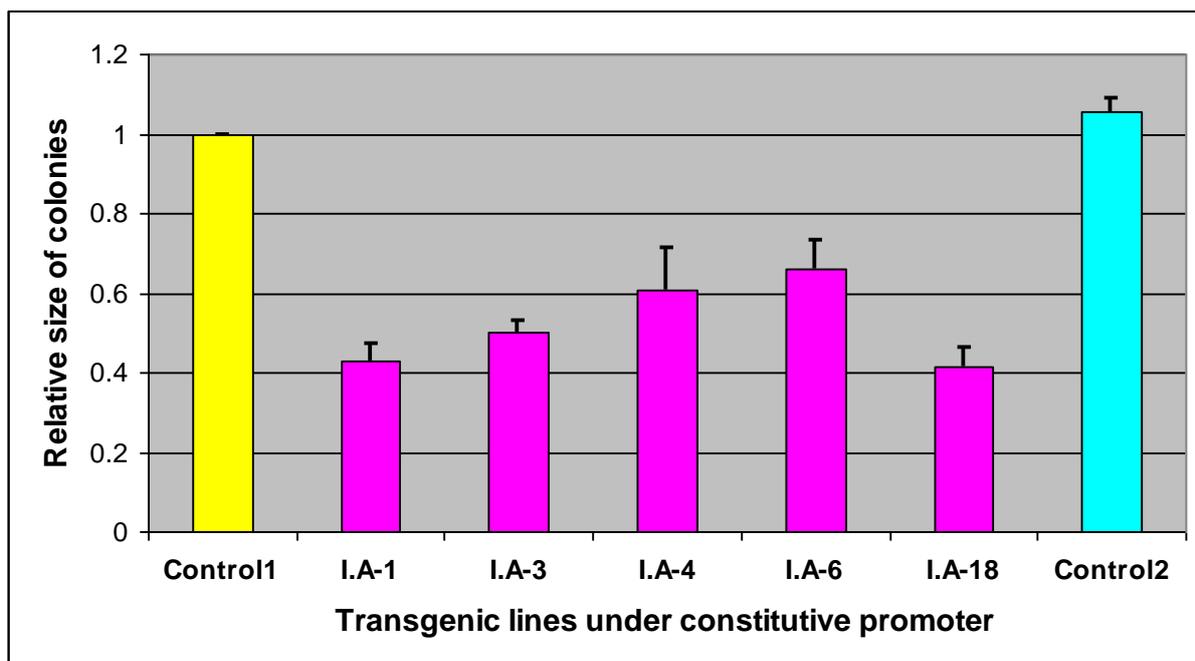


Figure: 3.19 Comparison of Colony size among Transgenics under Constitutive Promoter

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4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm². Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. First data were taken 9dpi for the number of developing colonies per leaf segment compared to the non transgenic control (shown in figure 3.18). The second data were taken 21 dpi on the colony size in terms of mm² and the average size on control and transgenic lines was compared

Figure: 3.19 represent the relative colony size of the transgenic plants (having *HarChit* and/or *HarCho* expressed under constitutive promoter) compared to the non transgenic and transgenic controls. Here control-1 is the non transgenic control while control-2 is the transgenic control with *gus* and *pat* constructs. The rest bars represent transgenic plants (I.A-1, I.A-3, I.A-3, I.A-4, I.A-6 and I.A-18)

Three weeks after inoculation the data were collected for the size of colonies developing on all the lines. The results are shown in Figure 3.19 the size of the randomly selected colonies was measured in terms of square millimetres and then compared with the Control1. The results showed a less growth in the size of the colonies growing on the transgenic lines for *HarChit* and *HarCho* under *Ubiquitin* promoter as the size of colonies on Control2 was comparable to non transgenic control. There was 34 to 60 percent less fungal growth on tested transgenic lines compared to control lines. Even the line I.A-1 that expressed only *HarChit* showed reduction. It also seems from these experiments that there is no role of *bar* or *gus* genes for fungal resistance. Figures 3.20, 3.21 and 3.22 show the examples of the real experiments. 3.20 is the control plate where cut leaf segments cultured on anti-senescence media were kept with the inoculated plates containing transgenic and control probes. 3.21 shows the normal view of the plate with I.A-6 and Control1 three weeks after inoculation and 3.22 is the close view of the same plate. From all these three figures it is clear that whenever there is no inoculation there is no senescence of leaves or very less senescence and when there is artificial inoculations there is less fungal development on the I.A-6 (constitutive expression of *HarChit* and *HarCho*) transgenic line and a lot more fungal development on non transgenic control. The results are clearer in 3.22.

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Figure: 3.20 none inoculated control three weeks after culture on the anti-senescence media.

4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm². Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. On control plates the leaf segments were cultured but no PM material was blown. These plates were also under the same conditions as experimental plates. There is no senescence or development of any pathogen 3 weeks after culture at the control plates.

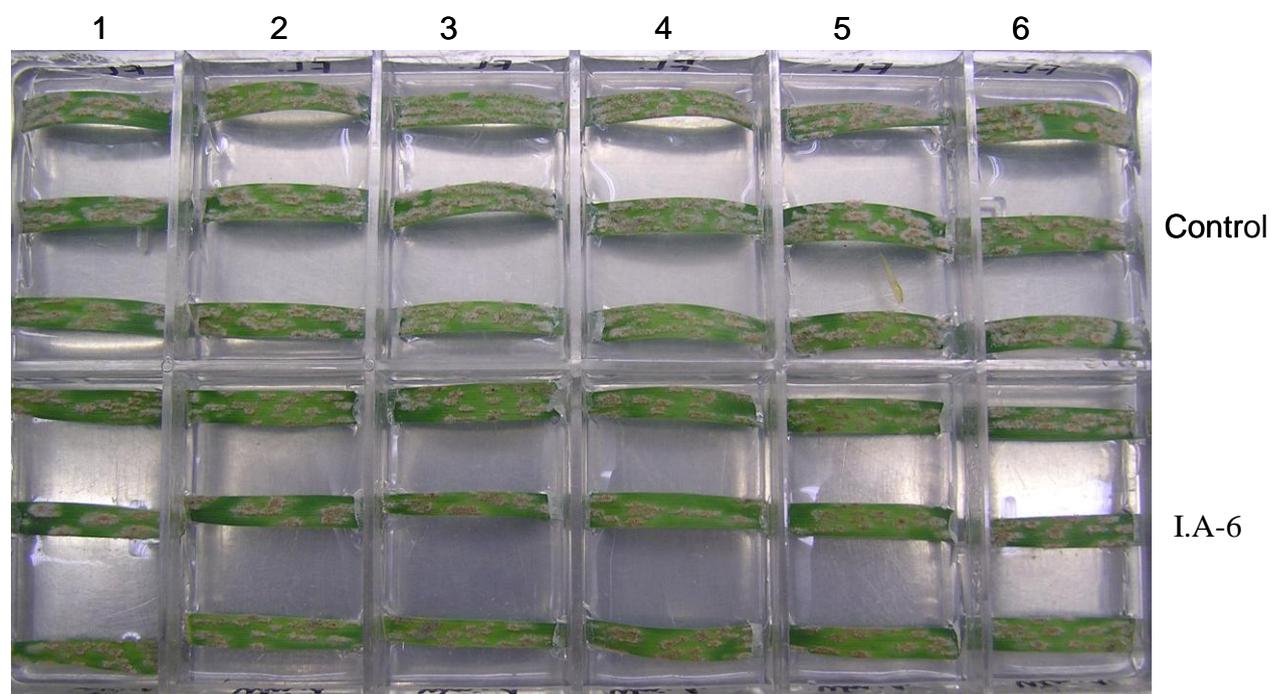


Figure: 3.21 Experimental sample of PM inoculation on over expression transgenic line I.A-6

4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm². Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. First data were taken 9dpi for the number of

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developing colonies per leaf segment compared to the non transgenic control (shown in figure 3.18). The second data were taken 21 dpi on the colony size in terms of mm^2 and the average size on control and transgenic lines was compared

Figure: 3.21 show the real picture of the experiment 21 dpi. 1-6 are repeats used per experiment. Control is non transgenic line Florida used as negative control. Figure shows the development of *E. graminis* colonies on control and transgenic line I.A-6 21 dpi.

The results show a decrease in the number and size of PM colonies developing on the lines with over expressed *HarChit* and *HarCho* genes. The figure shows not only small colonies on the transgenic plants as compared to non transgenic control plants but there is also the presence of dead colonies. On non transgenic lines not only the colonies are more in number but also they are healthy and contain more powdery material.

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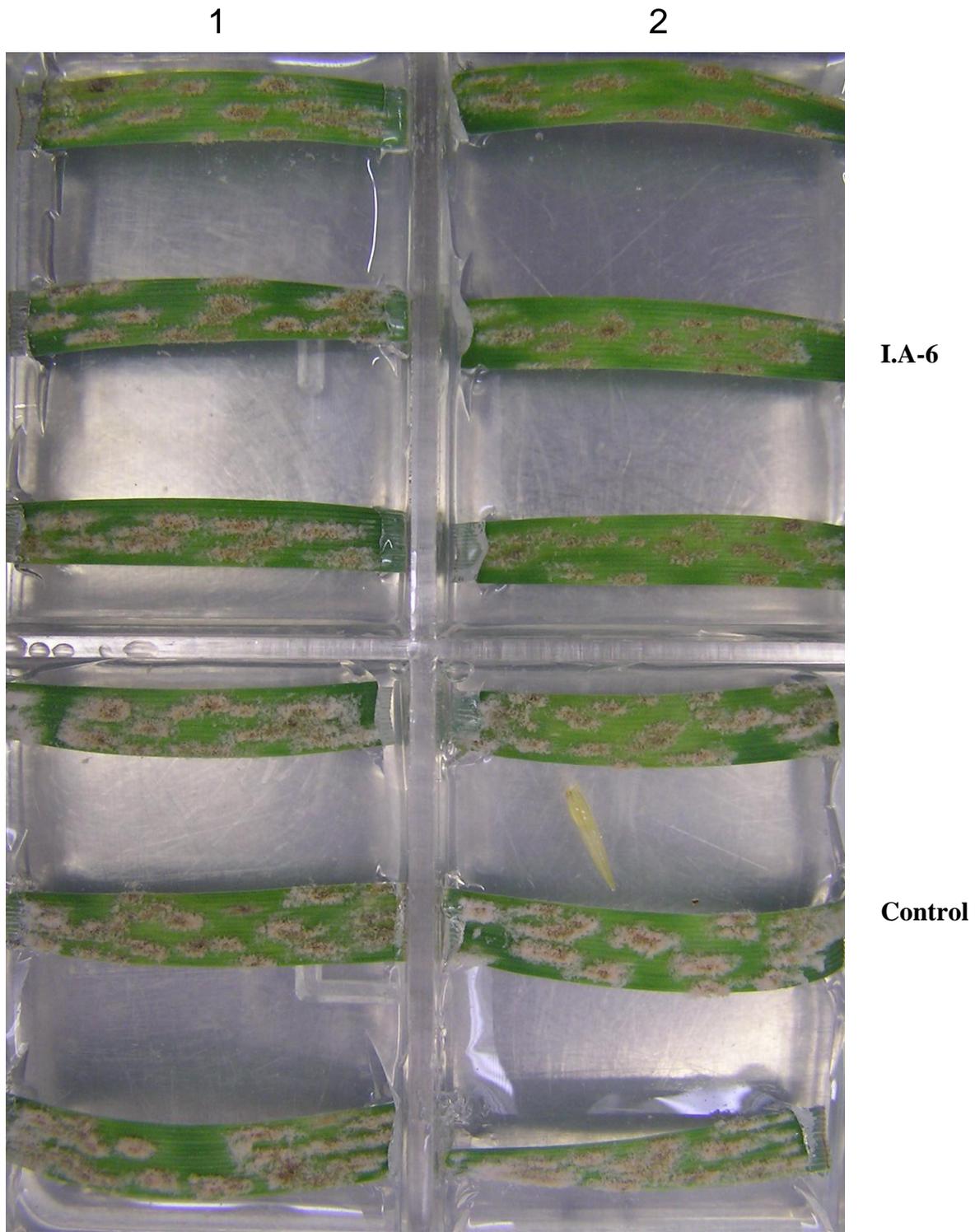


Figure: 3.22 Experimental sample of PM inoculation on over expression transgenic line I.A-6 (Closer view)

Figure 3.22 is the close view of the experiment presented in figure 3.21 and shows clear difference in the transgenic and non transgenic control in terms of resistance to *E. graminis*. Transgenic has completely controlled the pathogen after 3 weeks but the non transgenic could not.

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The results show that the expression of antifungal genes constitutively in wheat did not completely block the development and growth of the PM fungus but stopped the initial establishment to some and further spread to a great extent.

3.1.1.14.2 FHB (*Fusarium graminearum* L.):

Fusarium head blight is caused by *Fusarium graminearum* and related species of genera Fusarium. The pathogen causes disease in wheat, barley and related species of grass family. Only three lines out of the seven were checked for FHB. The inoculations were done according to the protocols explained in section 2.2.6.2.

The plants were transferred to the infection chamber at least a week before making inoculations.

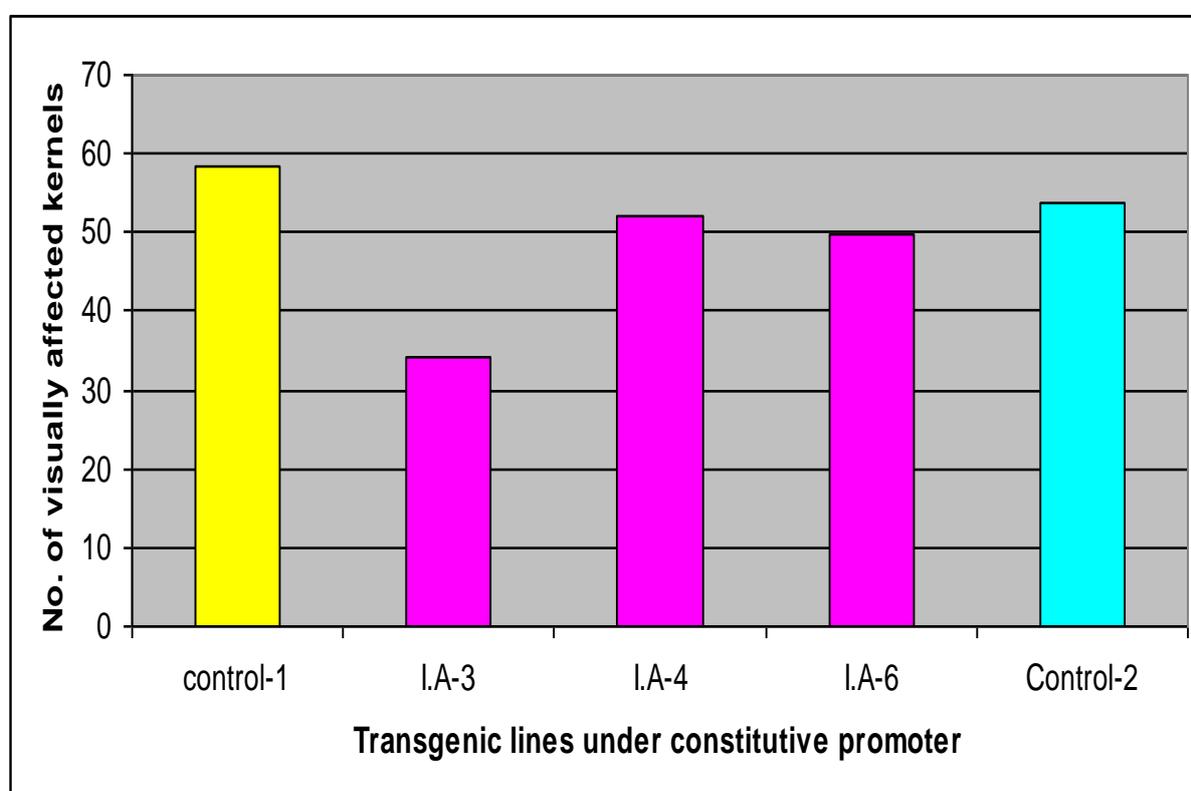


Figure: 3.23 Comparison Visually Effected Kernels among Transgenics under Constitutive Promoter

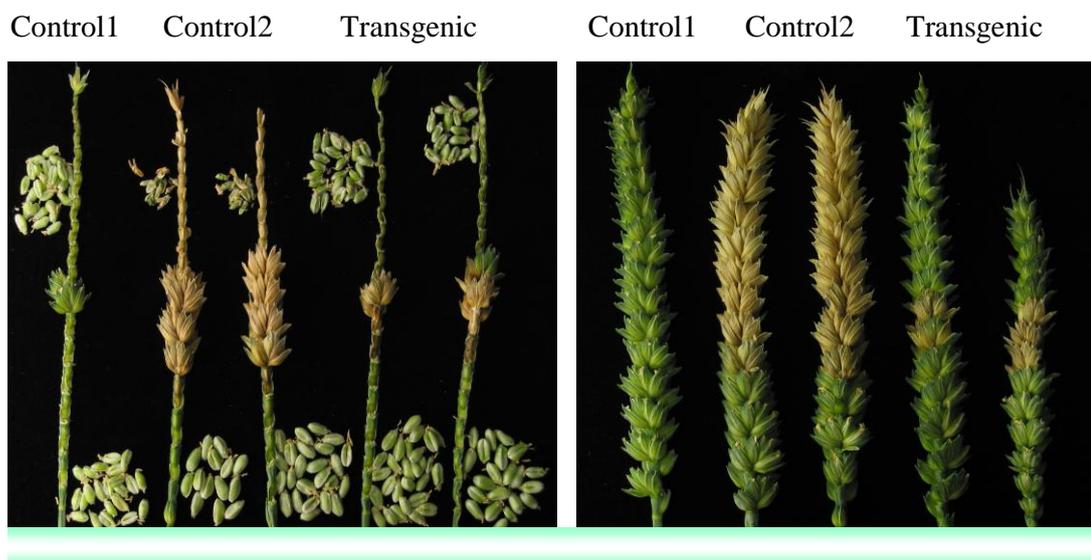
Figure: 3.23 show the percentage of the visually affected kernels 21 dpi in the non transgenic, transgenic controls along with transgenic lines (having *HarChit* and/or *HarCho* expressed under constitutive promoter).

The inoculations were made with 200 conidia per spikelet in the middle two spikelets just before fertilization. The experiment was kept at recommended condition for *F. graminearum* development stated in the section 2.2.6.2 of this manuscript. The data were taken on visually affected kernels 21 dpi.

The inoculations were made in the central two spikelets with 200 conidia in 10 μ L of water just before fertilization and the disease progression was followed in the adjacent spikelets for the next three weeks. The differences started getting clear one week after inoculations. The

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data was based on the percentage of the visually infected kernels. Out of the three lines checked transgenic line named I.A-3 showed the maximum reduction in the visually affected kernels with an average of 34.25% visually effected kernels while I.A-6 and I.A-4 showed comparatively less resistance to the disease with an average of 52.11% and 49.72% visually affected kernels. Non transgenic and transgenic control lines showed the highest percentage of visually affected kernels with an average of 58.25% and 53.75% respectively.



Control1 is H₂O inoculated spike, control2 is non transgenic inoculated control and transgenic is I.A-3 line.

Figure: 3.24 comparison of the visually affected transgenic (HarChit and HarCho under constitutive promoter) kernels and spikelets with wild type 21 dpi of *F. graminearum*.

Figure 3.24 compares the examples from real experiments done with transgenic plants (having *HarChit* and/or *HarCho* expressed under constitutive promoter) in comparison with non transgenic control and infection control. Figure 3.24 shows the response 21dpi. The inoculations were made with 200 conidia per spikelet in the middle two spikelets just before fertilization. The experiment was kept at recommended condition for *F. graminearum* development stated in the section 2.2.6.2 of this manuscript. It shows that *F. graminearum* travels through rachis of the spike. In transgenic spikes it looks to reach rachis but could not reach the spikelets.

The figure 3.24 shows an example of the infection process in both control (H₂O inoculated and pathogen inoculated) as well as in the over expression transgenic lines under *Ubiquitin* promoter. It is evident here that non transgenic control offers more spikelets for propagation while transgenic lines offer comparatively less spikelets for propagation. In control plants the nutrient supply is cut sooner and the upper part is dead leading to shrivelled and dead kernels.

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3.1.1.15 Pathological Testing of the transgenic plant with over expression of *HarChit* and *HarCho* under stress inducible promoter:

Wheat pathogens '*Erysiphe graminis* f.sp. *tritici*' and *Fusarium graminearum* were used for the evaluation of disease resistance capacity of the over expression transgenic plants under inducible promoter as were used for transgenics under constitutive promoter.

PM (*Erysiphe graminis* f.sp. *tritici*) infection studies:

The over expression transgenic lines under inducible promoter were also inoculated with PM causal organism as were done with the transgenics under constitutive promoter. The inoculation protocols are explained in the section 2.2.6.1. It was made sure to spread 300-400 conidia per centimetre square of inoculation plates. The infection plates were always covered to stop the contamination with other pathogens and ensure high relative humidity for fungal growth. The data were collected on number of germinating colonies and colony size nine days and three weeks post inoculation respectively.

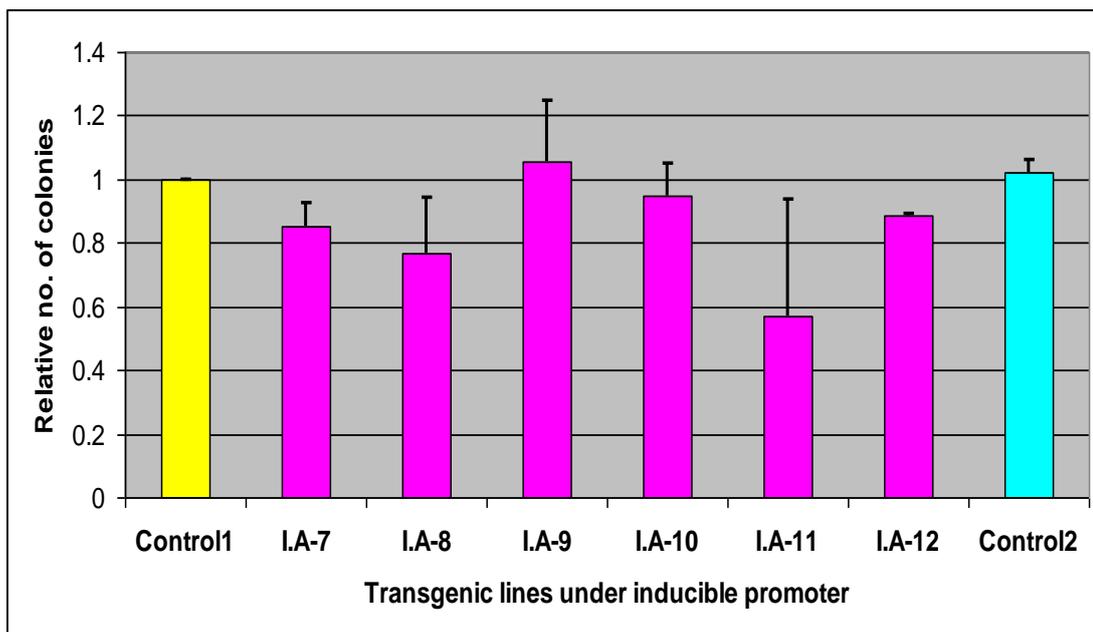


Figure: 3.25 Comparison for the number of colonies per leaf disk among Transgenics under Inducible Promoter

4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm². Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. First data were taken 9dpi for the number of developing colonies per leaf segment compared to the non transgenic control.

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Figure: 3.25 shows the relative number of colonies developed on the leaf disks of the transgenic plants (having *HarChit* and/or *HarCho* expressed under stress/disease inducible promoter) compared to the non transgenic and transgenic control plants. Here control-1 is the non transgenic control while control-2 is the transgenic control with *gus* and *bar* constructs. The rest bars represent transgenic plants.

The data collected for the number of colonies developing on the young leaf segments was taken nine days post inoculations and an average number of developing colonies per leaf segment were calculated. The averages of all the individual transgenic lines were then compared with non transgenic control. The results showed that all the lines had different average number of colonies after nine days of inoculations. Control2 (transgenic control) had 1.03 colonies as compared to every colony on control1 (non transgenic control). All the other transgenic lines had less number in comparison to non transgenic control except line I.A-9 who had 1.14 colonies in comparison to every colony found on the non transgenic control. Sizes of the selected developing colonies were measured three weeks post inoculations in terms of millimetres square and an average was calculated for every individual line. These averages were then compared taking the non transgenic control line colony size as one.

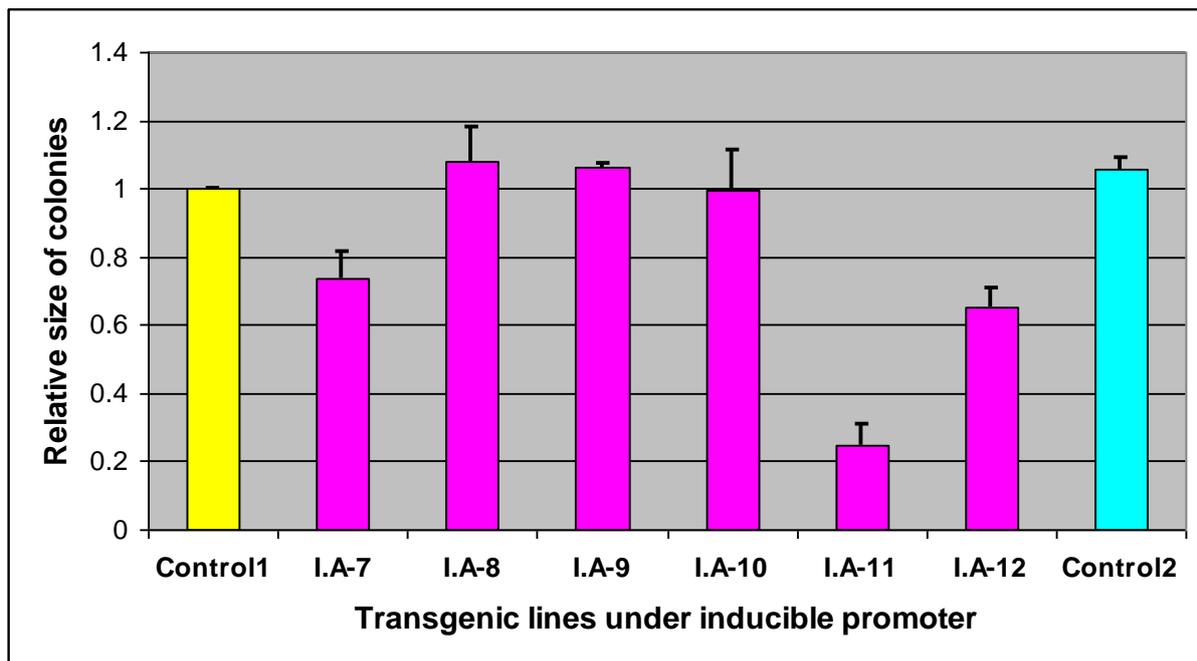


Figure: 3.26 Comparison of Colony size among Transgenics under Inducible Promoter

Figure: 3.26 represent the relative colony size of the transgenic plants (having *HarChit* and/or *HarCho* expressed under inducible promoter) compared to the non transgenic and transgenic controls. Here, control-1 is the non transgenic control while control-2 is the transgenic control with *gus* and *pat* constructs. The rest bars represent transgenic plants. The data were taken 21days after inocution with 300-400 conida per cm². The results indicate that some transgenic plants which are relatively more susceptible than the controls while the others are visibly less susceptible than the negative controls.

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The data collected for colony size is presented above. It is evident from here that a detectable reduction in colony size was observed in three out of seven lines under the test. Among these lines I.A-11 showed the most reduction. If non transgenic line produced an average colony size of 1 millimetre I.A-11 colony size was 0.25 millimetre. Lines I.A-7 and I.A-12 also showed good reduction in colony size with 0.74 and 0.64 millimetres in comparison to a colony size of 1 millimetre for non transgenic control. Non transgenic control, line I.A-10 and transgenic control lines produced almost the same size while the colony size was even bigger than the controls in lines I.A-8 and I.A-9.

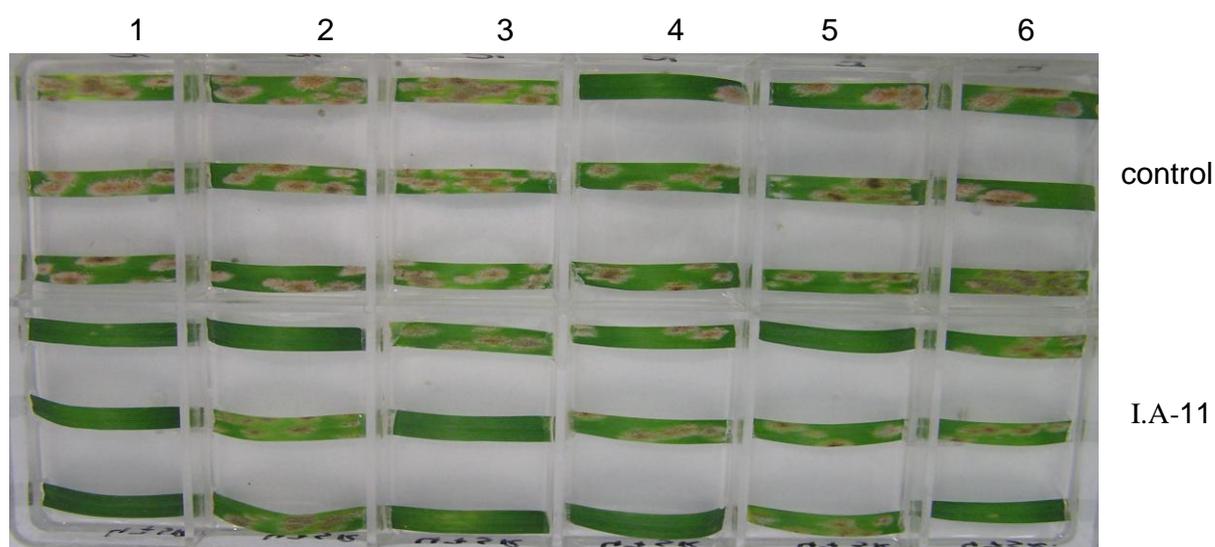


Figure: 3.27 Experimental sample of PM inoculation on over expression transgenic line I.A-11

4 cm long young leaf segments of all the transgenic plants were cultured separately on anti senescence media in comparison with non transgenic leaf segments and powdery material was blown onto them ensuring 300-400 conidia per cm^2 . Transgenic control (with *gus* and *bar* genes) was also compared with non transgenic control. The experiment was kept under ideal conditions for fungal growth mentioned in the section 2.2.6.1 of this manuscript. First data were taken 9dpi for the number of developing colonies per leaf segment compared to the non transgenic control (shown in figure 3.18). The second data were taken 21 dpi on the colony size in terms of mm^2 and the average size on control and transgenic lines was compared

Figure: 3.27 show the real picture of the experiment 21 dpi. 1-6 are repeats used per experiment. Control is non transgenic line Florida used as negative control. Figure shows the development of *E. graminis* colonieas on control and transgenic line 'I.A-6' 21 dpi.

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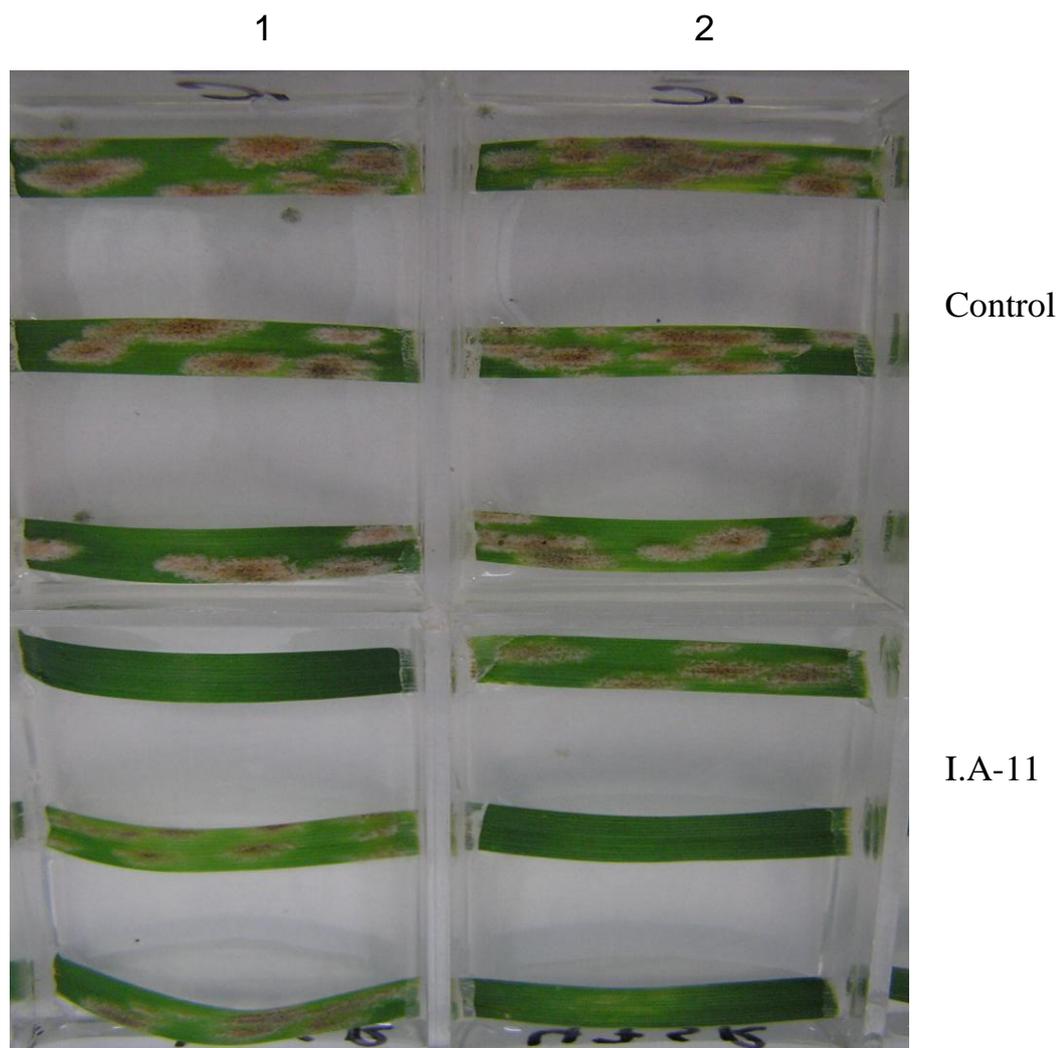


Figure: 3.28 Close view of Experimental sample of PM inoculation on over expression transgenic line I.A-11

Figure: 3.28 close view of the experiment presented in figure 3.27 shows clear difference in the transgenic and non transgenic control in terms of resistance to *E. graminis*. Transgenic has completely controlled the pathogen after 3 weeks but the non transgenic could not.

The figures 3.27 and 3.28 above show real examples of the same experiment that shows not only the reduction in the number of the PM colonies per leaf segment but also the deterioration of the health of the colonies. Control leaf segments showed healthy colonies with a lot of mycelia while transgenic leaf segments had fewer colonies and all the mycelia were also dead. The transgenic leaf segments also seemed to have more plant cell death shown in terms of more yellowness.

FHB (*Fusarium graminearum*) inoculations:

F. graminearum is a pathogen of the wheat head. Two central wheat spikelets were inoculated with 200 conidia/10 μ L of water at just before anther maturity stage. The infection process

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was followed for three weeks. The differences started to become clear 9 days after inoculations.

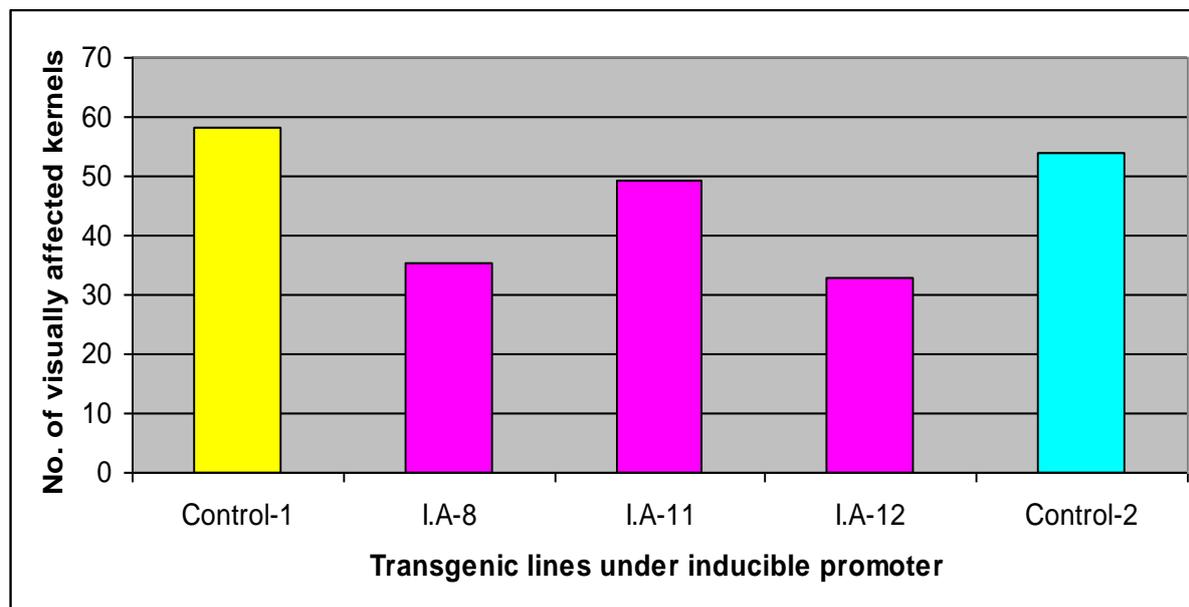


Figure: 3.29 Comparisons of Visually Effected Kernels among Transgenics under Inducible Promoter

Figure: 3.29 show the percentage of the visually affected kernels 21 dpi in the non transgenic, transgenic controls along with transgenic lines (having *HarChit* and/or *HarCho* expressed under stress/disease inducible promoter). The inoculations were made with 200 conidia per spikelet in the middle two spikelets just before fertilization. The experiment was kept at recommended condition for *F. graminearum* development stated in the section 2.2.6.2 of this manuscript

Three weeks after inoculations the data were taken on the number of affected kernels. Only three transgenic lines were checked for their reaction against *F. graminearum*. The data were taken and the percentage of the visually effected kernels was calculated on all the individual transgenic lines as well as control lines. The data presented above depicts that the control lines showed highest percentage of the effected kernels with a percentage of 58.25 and 53.75 for non transgenic and transgenic controls respectively. Out of the three lines tested I.A-12 showed the least visually effected kernel with a percentage of 32.75 effected kernels. I.A-8 and I.A-11 also showed a detectable reduction of visually effected kernels with 35.30% and 49.26% of visually effected kernels respectively.

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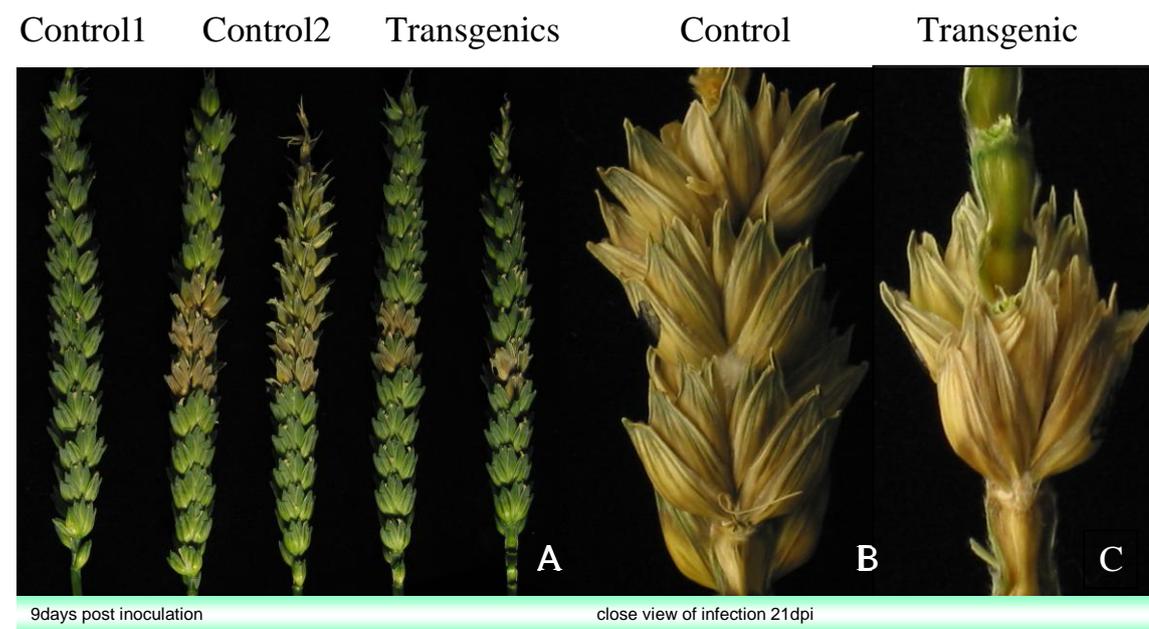


Figure: 3.30 Visual examples of FBH experiments.

Figure: 3.30A= Comparison of water control, non transgenic control and transgenic line I.A-12, 9 days after inoculation, B= Infection area of the non transgenic control 21 dpi. C=Infection area of transgenic (I.A-12) 21 dpi

Figure 3.30 shows the examples of the general trends in the pathology experiments and reveals how the transgenic lines resist against the pathogen and stop it from spreading to the next spikelets.

3.1.2 Down regulation/Knock Down of *TaGSL* Genes by PTGS:

Three genes were selected for the evaluation of their function by knocking their expression down through post transcriptional gene silencing. Knock down/RNAi constructs were prepared by cloning selected small fragments from the concerned genes in opposite orientation under *Ubiquitin* promoter and *nos* terminator. The fragments were separated by *gus* spacer. The constructs were named as *pRNAi-GSL-3*, *pRNAi-GSL-8*, *pRNAi-GSL- 8'* and *pRNAi-GSL-10*. The details about these constructs can be seen in section 2.2.3.1.

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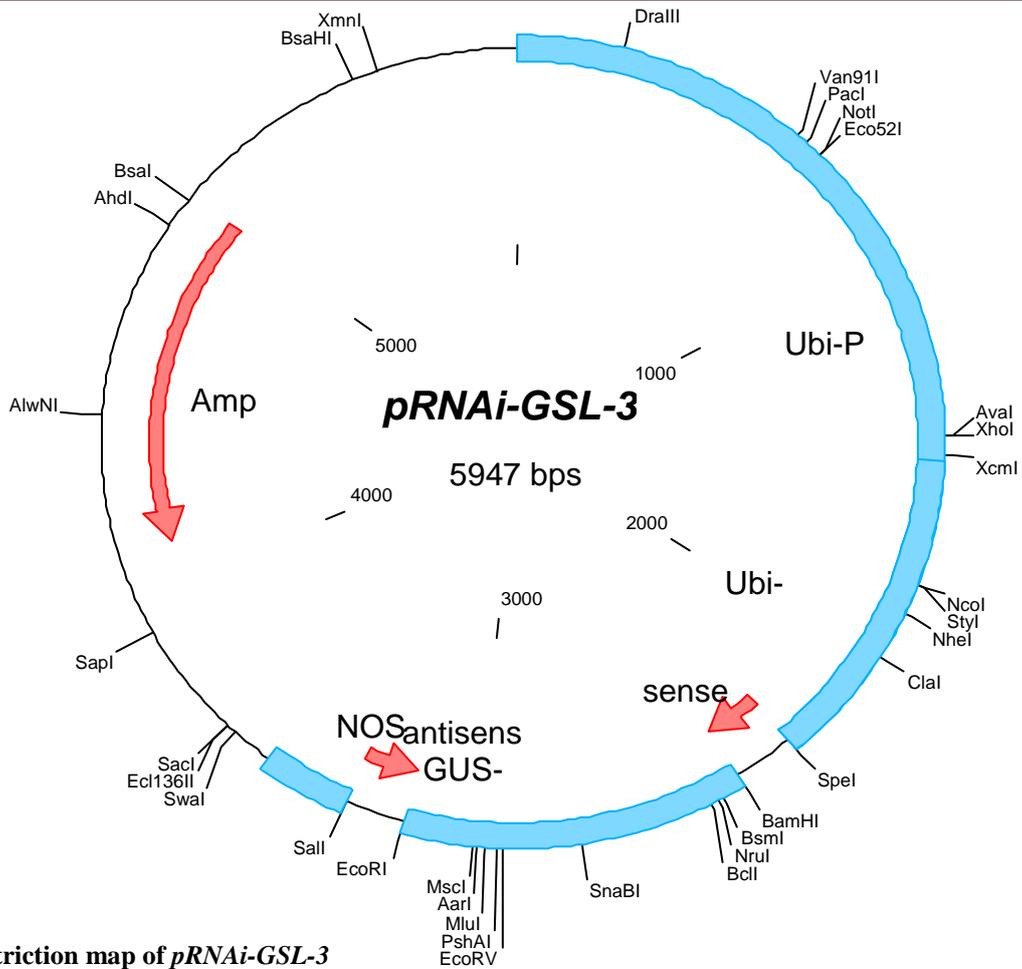


Figure 3.31. Restriction map of *pRNAi-GSL-3*

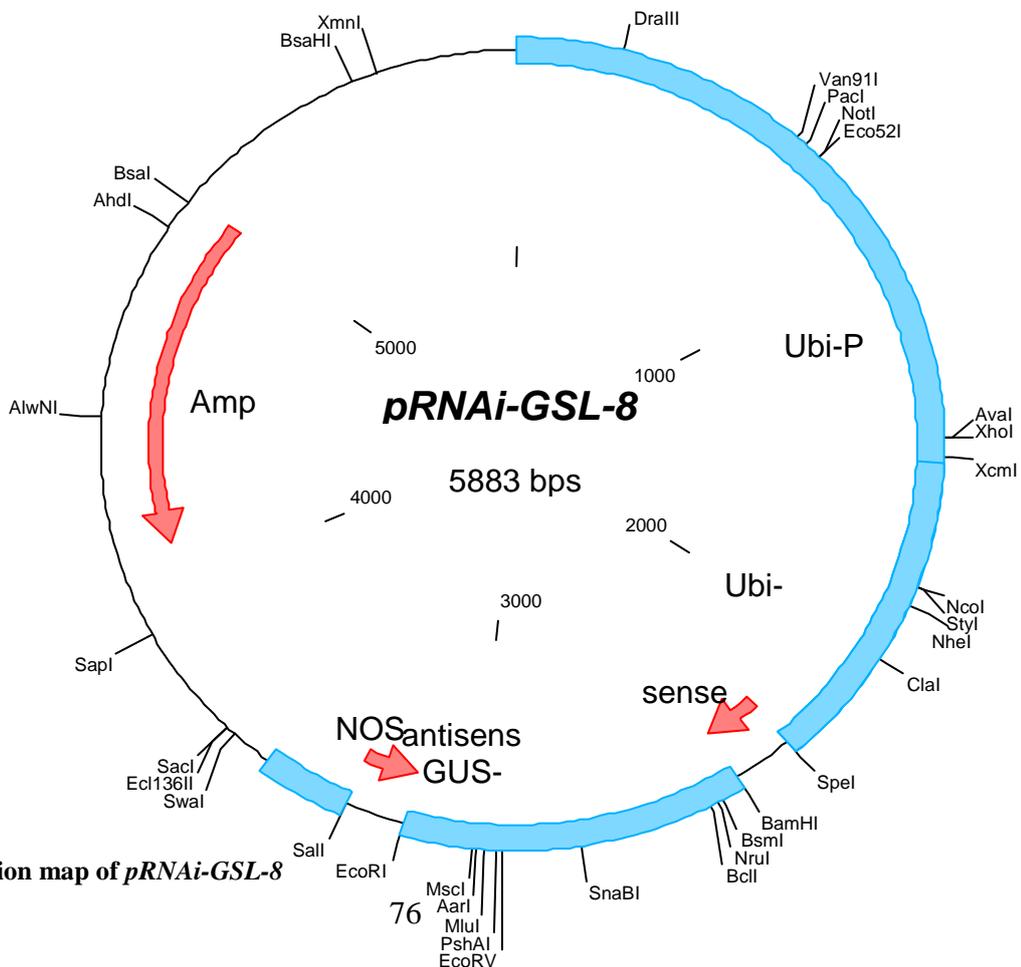


Figure 3.32. Restriction map of *pRNAi-GSL-8*

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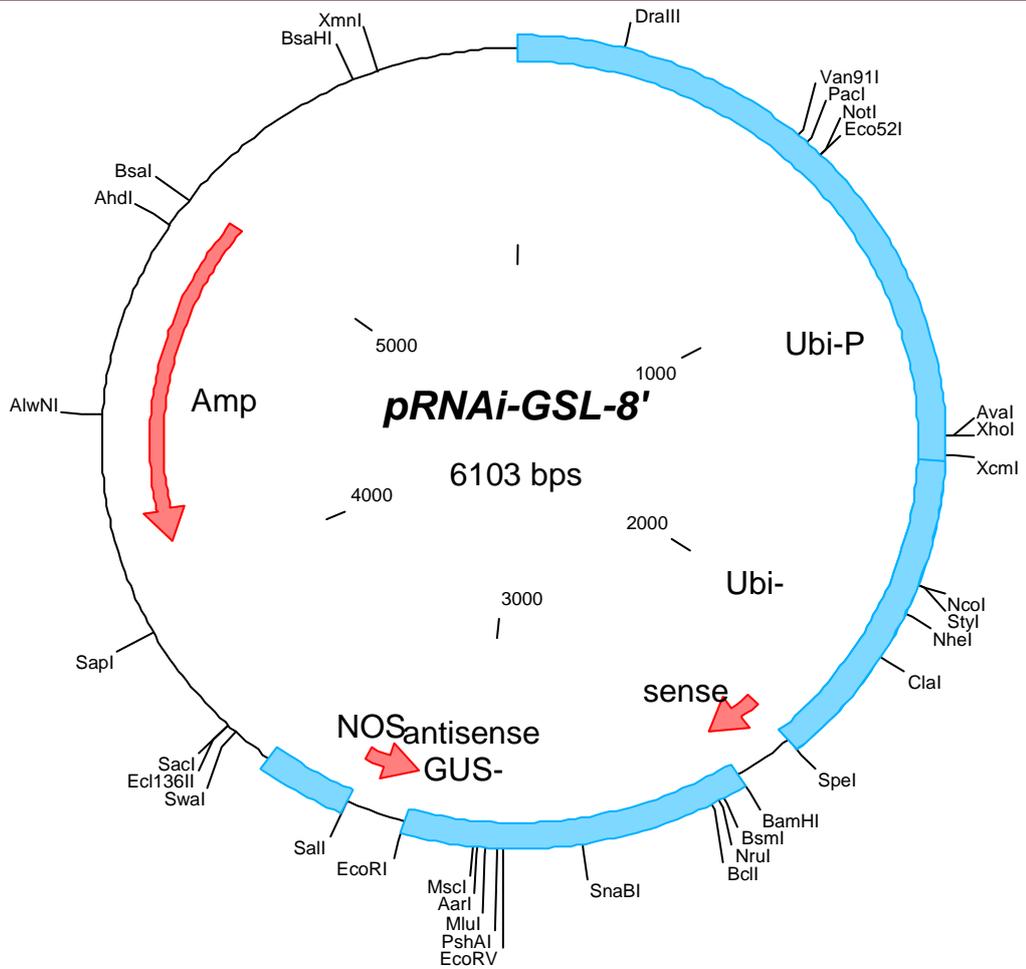


Figure: 3.33 Restriction map of *pRNAi-GSL-8'*

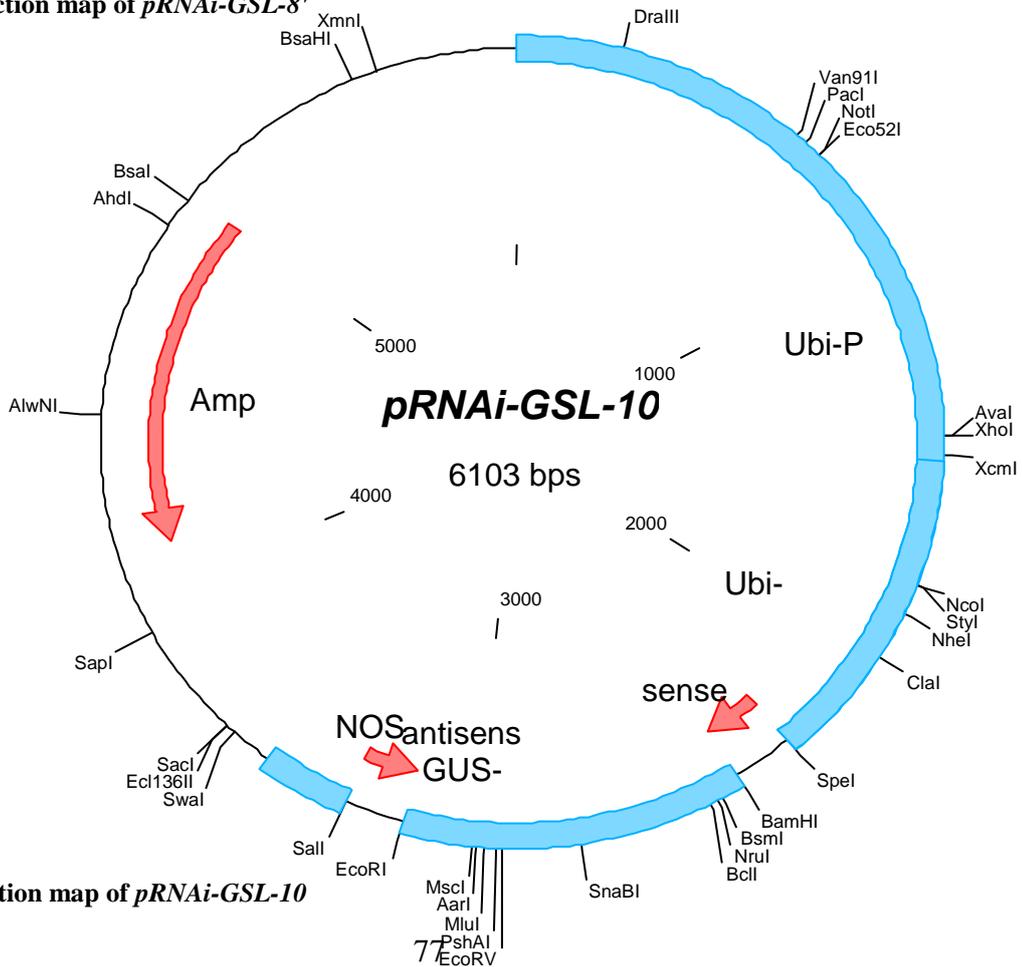


Figure: 3.34 restriction map of *pRNAi-GSL-10*

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Figures 3.30 to 3.34 show all the four RNAi constructs used for PTGS. The construct cards show some useful enzymes which can be used for restriction digest and give an over view of all the components.

3.1.2.1 Transformation with Knock down Constructs:

The constructs were introduced into IZEs using the protocols described earlier for transformation through biolistic bombardment as stated in section 2.2.4. A total of 21286 IZEs were bombarded with different RNAi constructs. The bombarded embryos were forwarded to the tissue culture media recommended by Becker *et al.* (1994) and *BASTA* resistant putative transformants were recovered around three weeks after bombardment. A total of eight putative transgenic plants could be recovered by final selection in the green house. Out of these 8 transgenics 3 belonged to *pRNAi-GSL-3* and 5 to *pRNAiGSL-8* while none of the plants were got for the rest of the knock down constructs. The results are better clarified in the table 3.4. The transformation frequency was calculated as zero or nearly zero.

Table: 3.4 Transformation Frequency with RNAi/PTGS constructs.

Gene constructs	No. of IZEs shots	Transgenic Plants recovered	Transformation percentage
pRNAi-GSL-3	7061	2	Nearly zero
pRNAi-GSL-8	6750	2	Nearly zero
pRNAi-GSL-8'	3725	-	-
pRNAi-GSL-10	3750	-	-

3.1.2.1.1 Determination of construct toxicity for knock down constructs:

In the wake of getting no transgenic plant for *pRNAiGSL-8'* and *pRNAi-GSL-10* it was decided to find out if the constructs were toxic for the development of transgenic plants. For that purpose 400 IZEs were bombarded only with selection marker gene *pat* (*p35SAcS*). At the same time 400 IZEs were bombarded separately with *pRNAiGSL-8'* and *pRNAi-GSL-10* along with *p35SAcS* as selection marker gene.

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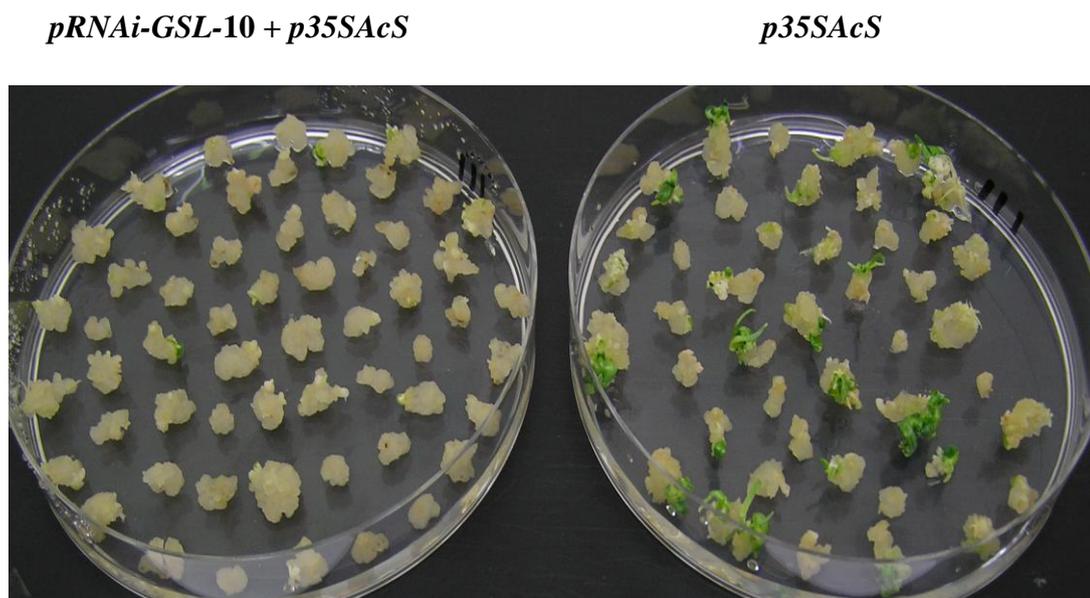


Figure: 3.35 Effect of *pRNAi-GSL-10* on regeneration frequency.

A set of 400 IZE's was shot with *pRNAi-GSL-10 + p35SAcS* and another set of 400 IZE's was shot with *p35SAcS* only and the embryos were put into the tissue culture process stated in the section 2.2.4.4 of this manuscript. Calli started developing normally on SM-I (two weeks) and continued on SM-II (Two weeks) and were transferred to SM-III for regeneration. Data were taken two weeks after the transfer of calli onto the SM-III.

Figure 3.35 shows the comparison of tissue culture stage SM-III for *pRNAi-GSL-10 + p35SAcS* (RNAi plus selection marker constructs) and *p35SAcS* alone (selection marker gene). Figure shows almost no embryo development when RNAi construct was used with the selection marker construct but there were a good number of embryos developing when only selection marker was used.

IZEs were put to tissue culture after bombardment. The number of regenerating embryos was calculated two weeks after putting the calli to the SM-III. Only 12 and 10 percent of the calli produced some green embryos in *pRNAiGSL-8'* and *pRNAi-GSL-10* respectively. On the same conditions 50 percent of the calli showed somatic embryo development in control bombardments with *p35SAcS*. The situation is better clear in figures 3.35 and 3.36 where the calli in the Petri plate which was bombarded by *pRNAi-GSL-10 + p35SAcS* or *pRNAi-GSL-8' + p35SAcS* show very less number of regenerating embryos and those too unhealthy. The plate which was bombarded by *p35SAcS* only showed visibly nice and healthy embryo production. It shows that the knock down constructs with 230 bp sense and antisense fragments were toxic for somatic embryo development. Somatic embryos found in the experiment were developed into plants and a final selection was done in the green house where all the plants from *pRNAiGSL-8'* and *pRNAi-GSL-10* died as seen earlier while four plants survived for the control construct.

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pRNAiGSL-8' + p35Sacs

p35Sacs

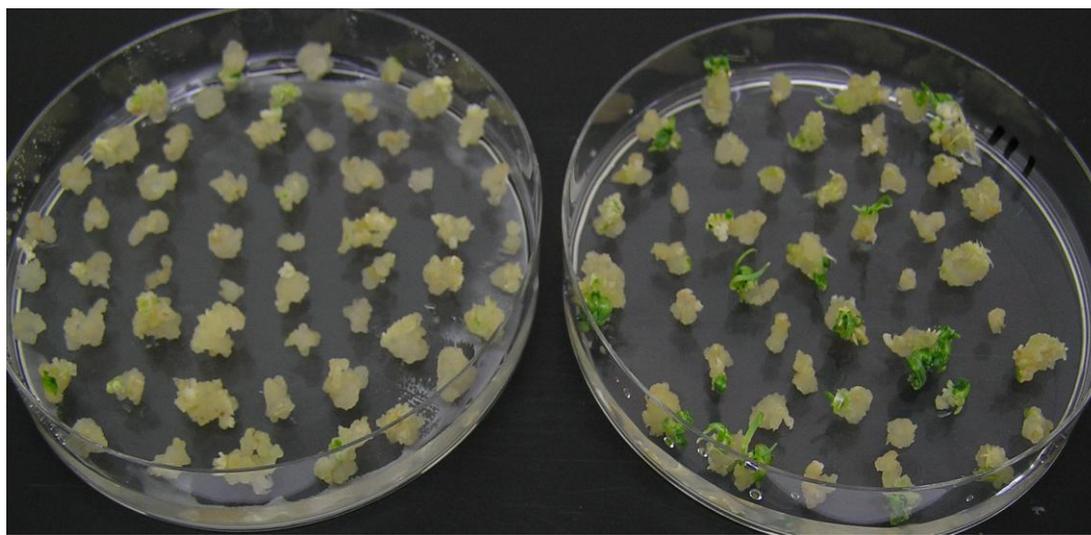


Figure: 3.36 Effect of *pRNAiGSL-8'* on regeneration.

A set of 400 IZE's was shot with *pRNAi-GSL-8' + p35Sacs* and another set of 400 IZE's was shot with *p35Sacs* only and the embryos were put into the tissue culture process stated in the section 2.2.4.4 of this manuscript. Calli started developing normally on SM-I (for two weeks) and continued on SM-II (for two weeks) and were transferred to SM-III for regeneration. Data were taken two weeks after the transfer of calli onto the SM-III medium.

Figure 3.35 shows the comparison of tissue culture stage SM-III for *pRNAi-GSL-8' + p35Sacs* (RNAi plus selection marker constructs) and *p35Sacs* alone (selection marker gene). Figure shows almost no embryo development when RNAi construct was used with the selection marker construct but there were a good number of embryos developing when only selection marker was shot.

3.1.2.1.2 Molecular Verification of knock down constructs in the transgenic plants:

The results of *BASTA* selection were further confirmed using Southern Blot analysis by DIG labelled probes which were able to bind in the spacer region of both the constructs. DNA was isolated from the leaves of all the putative transformants and digested with *KpnI* and *SfiI* endo-nucleases. The same was done with negative control non transgenic "Florida" DNA and positive control plasmid DNA. Two separate 0.8% gels were run for *pRNAi-GSL-3* and *pRNAi-GSL-8*. Three lanes were run for every plant probe with 25µg DNA per lane. First lane contained undigested DNA, second lane contained DNA digested with *KpnI* (linearizes the plasmid control) and third lane contained DNA digested with *SfiI* (cuts the RNAi cassette out of plasmid). Negative control lane was having DNA digested with *SfiI* while positive control was with linearization and cassette out. DNA was transferred to nylon membrane by capillary action and hybridization was done with DIG labelled probes, chemilluminescence's detection was done with CSPD® substrate and the pictures were taken on X-rays film.

RESULTS

pRNAi-GSL-3

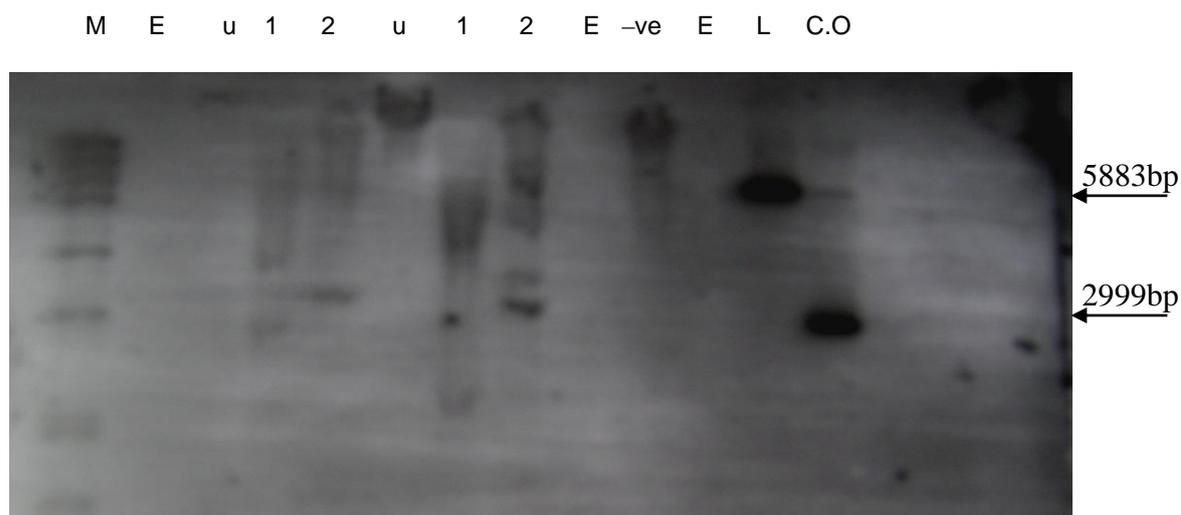


Figure: 3.37 Southern Blot analysis of TaGSL-3 knock down transgenics.

The digested and undigested g-DNA was loaded onto 0,8% agarose gel at the rate of 25 μ g DNA per lane. Plasmid control DNA was loaded at the rate of 25 pg along with 1 μ g of herring's sperm DNA to keep its velocity at par with the velocity of g-DNA. The gel was run and DNA was then transferred to nylon membrane by capillary action and cross linked. This DNA on the membrane was then hybridized with 400 bp long DIG labelled single stranded DNA specific to the *gus* spacer in the RNAi construct and the detection was done with CSPD substrate.

M = marker, E = empty, U = undigested g-DNA, 1 = DNA digested with *KpnI* that linearizes the RNAi plasmid. 2 = g-DNA digested with *Sfi-I* that cuts the cassette out of the plasmid vector. L= linearised plasmid DNA, C.O= plasmid DNA digested to cut the cassette out of the plasmid and -ve= non transgenic DNA digested with *Sfi-I*.

All the five putative transformants for *pRNAi-GSL-3* were checked by Southern Blot analysis and only two out of them were having the construct as is shown in figure 3.37. The plants were termed as Δ *TaGSL-3-1* and Δ *TaGSL-3-3*. Δ *TaGSL-3-1* seemed to have single copy integration and Δ *TaGSL-3-3* seemed to have multi copies of the construct.

RESULTS

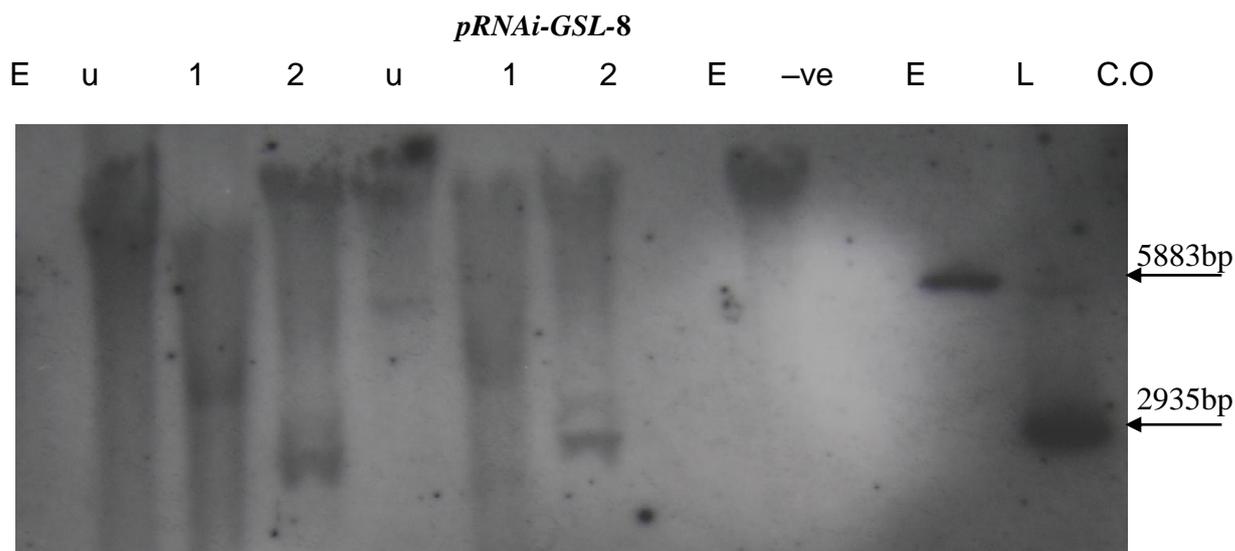


Figure 3.38 Southern Blot analysis of *TaGSL-8* knock down transgenics.

E = empty lane M = marker, E = empty, U = undigested g-DNA, 1 = DNA digested with *KpnI* that linearizes the RNAi plasmid. 2 = g-DNA digested with *SfiI* that cuts the cassette out of the plasmid vector. The digested and undigested DNA was run on the 0.8% and transferred to nylon membrane where RNAi positive plants were detected for *pRNAi-GSL-8* using the same protocols as stated in the figure. 3.37.

Five plants were recovered for *pRNAi-GSL-8* by final BASTA selection and two out of which were proved positive by Southern Blot analysis. They were named as $\Delta TaGSL-8-1$ and $\Delta TaGSL-8-3$. Both of them seemed to have one copy of the construct as is shown in figure 3.38.

3.1.2.1.2 Northern Blot analysis:

The functionality of the concerned constructs was checked by Northern Blot analysis in all the four plants. RNA was isolated from all plants proved to contain RNAi constructs along with non transgenic control and 15 μ g RNA per plant was run on 1% degeneration agarose gel. RNA was transferred to positively charged nylon membrane by capillary action. Hybridisation was done with radio actively labelled single stranded DNA fragment that binds to the *gus* spacer. Northern Blot analyses showed that all four plants had functional constructs and are producing the transcript to start knock down activity (Figure 3.36).

All the four plants of knock down from *TaGSL* family were advanced to the next generation and analysed there for the presence of the knock down constructs as well as their functionality by Northern Blot analysis in T₁ generation.

RESULTS

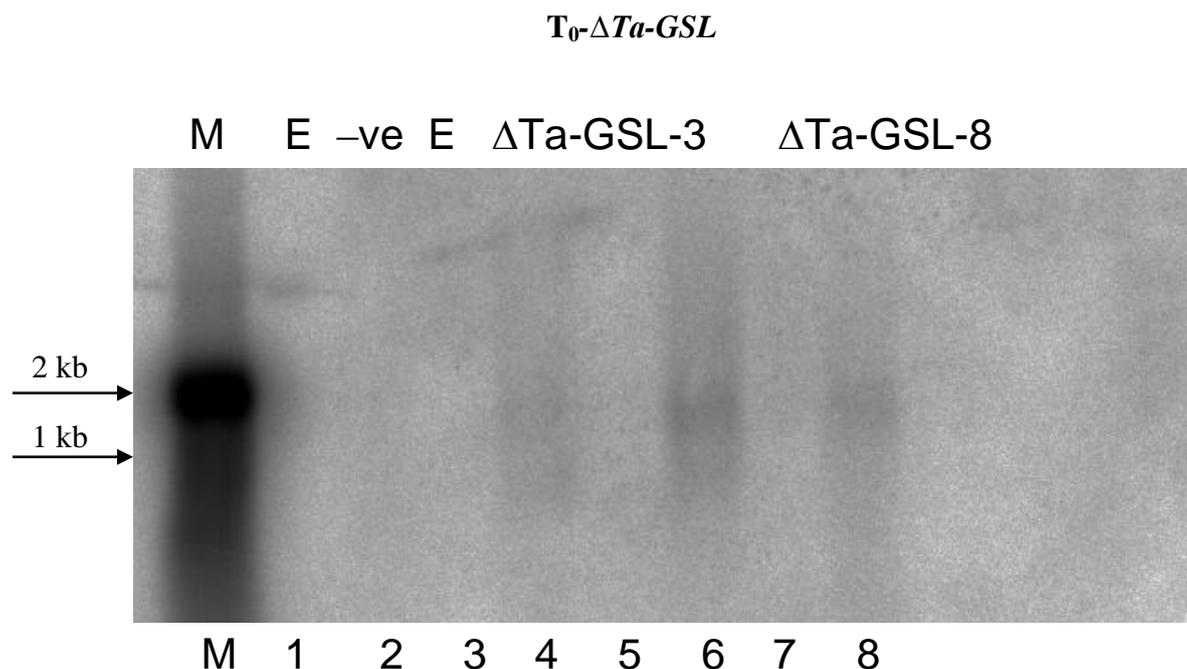


Figure: 3.36a Northern Blot analysis of T₀ ΔTa -GSLs

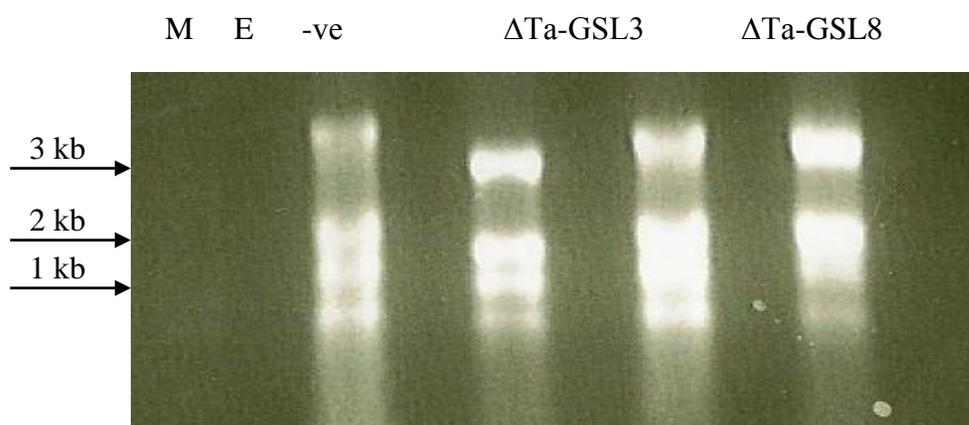


Figure: 3.36b. Control Gel for Northern Blot analysis of T₀ ΔTa -GSLs

Freshly isolated RNA from immature spike tissues from confirmed transgenic T₀ plants along with non transgenic control plant RNA was run on 1% degradation gel at the rate of 15 μ g per probe. This RNA was later transferred to nylon membrane and hybridized with radio actively labelled 400 bp long single stranded DNA specific to the *gus* spacer. The detection was made on the X-rays film which confirmed the expression of RNAi construct in all the four transgenic plants.

M = Marker, E = empty lane, -ve = non transgenic control, ΔTa -GSL-3 = knock down lines for *Ta-GSL3* gene, ΔTa -GSL-8 = knock down line for *Ta-GSL8* gene.

3.1.2.1.3 Molecular characterisation of ΔTa GSL knock Down Lines:

Self pollinated seeds were harvested from the knock down plants and T₁ generations were grown for all the four lines. Molecular characterisation was done before checking any role of these genes in disease resistance.

RESULTS

3.1.2.1.3.1 Southern Blot analysis for $\Delta TaGSL$ knock down lines:

Southern Blot analysis was done of the selected plants from all the four lines using protocols mentioned in 2.2.5.5. DNA was isolated from the selected plants of each line. DNA was digested with *SfiI* and 25 μ g per lane and per plant was run on 0.8% agarose gel separately for each line. Positive and negative controls were also run along with. DNA was later transferred to nylon membrane by capillary action and hybridised with radio actively labelled probe specific for *gus* spacer. The results were taken on X-rays film. Figure 3.37 presents one example of T₁ generation.

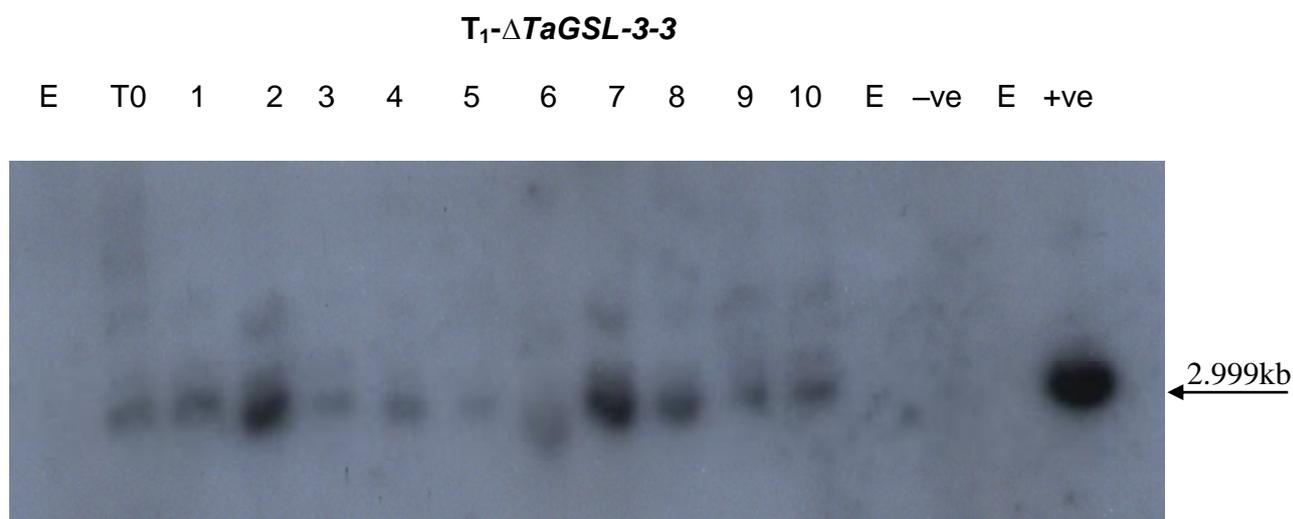


Figure: 3.37 Southern Blot analysis of T₁ - $\Delta TaGSL$

Digested DNA from primary transformant as well as from all the ten T₁ lines was run on the 0.8% agarose gel along with negative and positive plasmid control, transferred onto nylon membrane and hybridized with DIG labelled 400 bp long single stranded DNA fragment specific to the *gus* spacer of the RNAi construct. Detection was done by CSPD substrate and the results were taken on X-rays film that confirmed the transfer of RNAi constructs to the next generation.

E = empty lane, T₀ = primary transformant, 1-10 = the plants belonging to the T₁ generation, -ve = non transgenic DNA digested with *SfiI* (enzyme that cuts the expression cassette out of the plasmid vector) +ve = plasmid vector digested with *SfiI*

The results showed the successful transfer of knock down constructs to the next generation. The integration pattern was also the same as was seen in the T₀ generation of all the four plants.

3.1.2.1.3.2 Northern Blot analysis for $\Delta TaGSL$ knock down lines:

For the Northern Blot analysis the hybridization probe was specific to the siRNA sequences used in the knock down constructs. Two plants which were positive for constructs were selected from each line to check in the T₁ generation. The protocols were used as stated in the

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section 2.2.5.6. RNA was isolated from the immature spikes just before anthesis and 15µg RNA of each plant was run on 1% denaturing agarose gel and then transferred to the positively charged nylon membrane where it was hybridized with the radio actively labelled single stranded DNA molecules and the results were taken on the X-rays films.

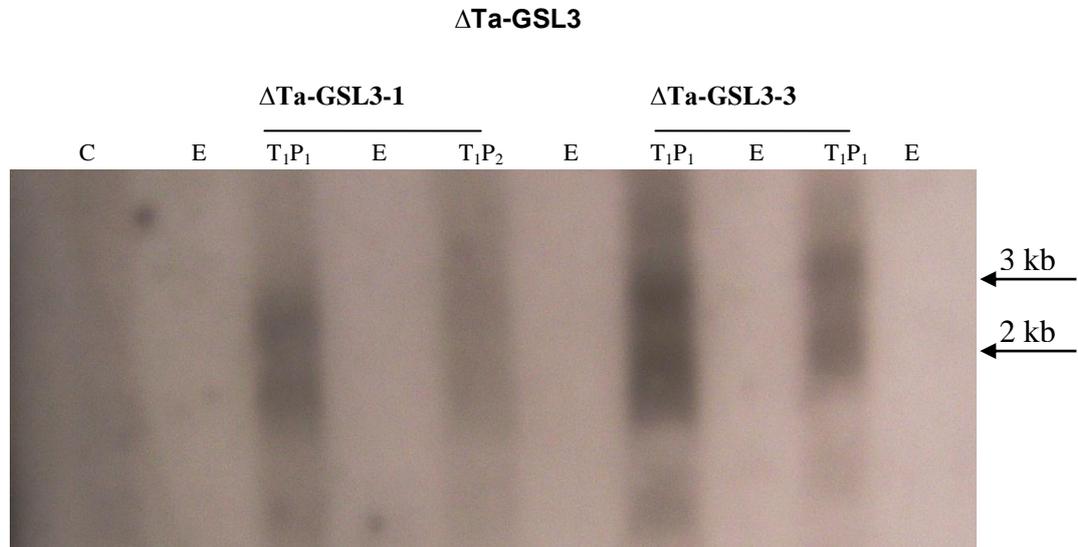


Figure: 3.38a Northern Blot analysis of the T₁ generation ΔTa-GSL3 plants.



Figure: 3.38b Control RNA Gel for Northern Blot analysis of the T₁ generation ΔTa-GSL3 plants.

Freshly isolated RNA from immature spike tissues from confirmed transgenic T₁ plants along with non transgenic control plant RNA was run on 1% degradation gel at the rate of 15 µg per probe. This RNA was later transferred to nylon membrane and hybridized with radio actively labelled 400 bp long single stranded DNA specific to the siRNA forming DNA fragment. The detection was made on the X-rays film which confirmed the expression of RNAi construct in all the selected plants in the T₁ generation.

M = Marker, E = empty lane, ΔTa-GSL-3-1 and ΔTa-GSL-3-3 = knock down lines for Ta-GSL3 gene.

The results showed that the large amount of the 150 bp hairpin forming RNA specific to both the *TaGSL-3* and *TaGSL-8* was produced as compared to same transcript produced by the normal non transgenic “Florida” genotype as can be seen in figure 3.38. It means that the constructs are active and creating the knock down of the concerned genes.

RESULTS

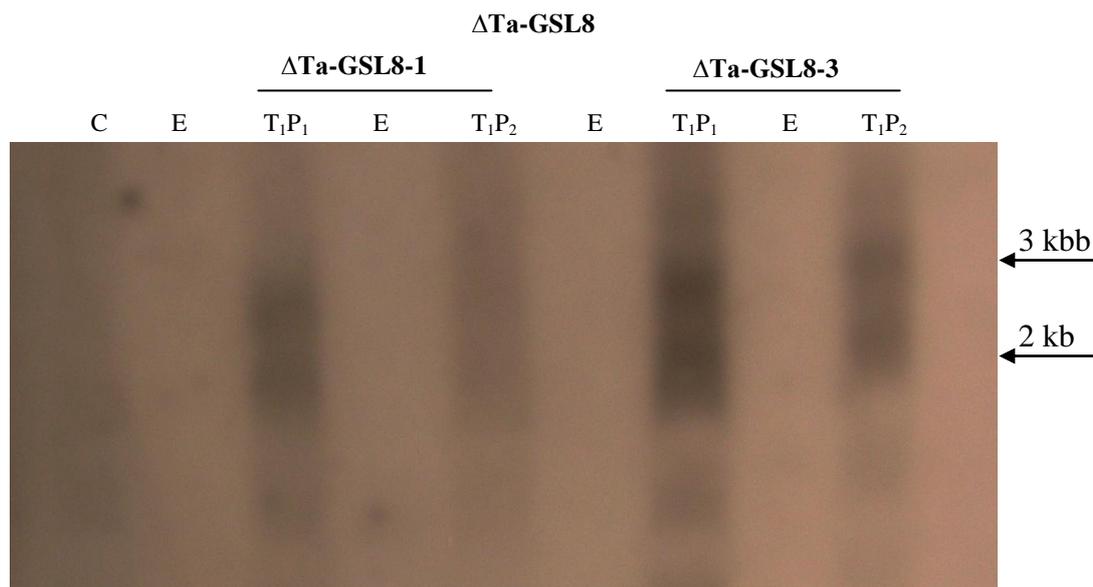


Figure: 3.39a Northern Blot analysis of the T₁ generation ΔTa-GSL plants.

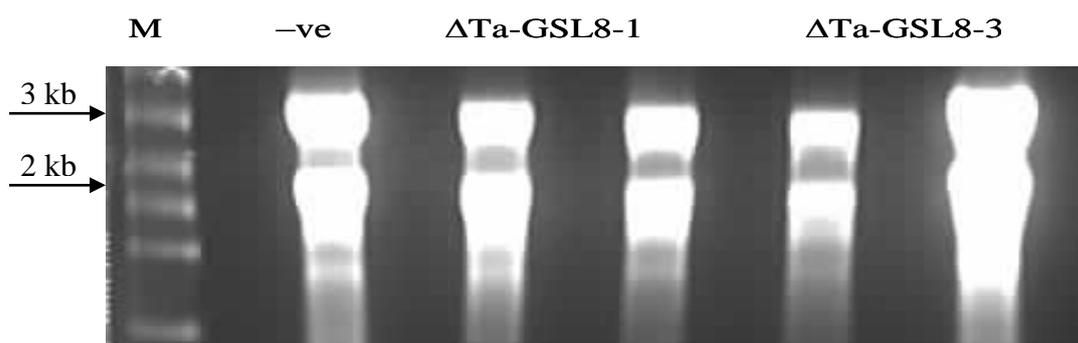


Figure: 3.39b Control gels for Northern Blot analysis of the T₁ generation ΔTa-GSL plants.

Freshly isolated RNA from spike tissues from confirmed transgenic T₁ plants along with non transgenic control plant RNA was run on 1% degradation gel. This RNA was later transferred to nylon membrane and hybridized with radio actively labelled 400 bp long single stranded DNA specific to the siRNA forming DNA fragment. The detection was made on the X-rays film which confirmed the expression of RNAi construct in all the selected plants in the T₁ generation.

M = Marker, E = empty lane, Δ*Ta-GSL-8-1* and Δ*Ta-GSL-8-3* = knock down lines for *Ta-GSL8* gene.

As can be seen in the figures 3.38 and 3.39 the most of the transcript was accumulated around two heavier bands but in general the results look like a smear although the control RNA gels did not show a lot of degraded RNA. Non transgenic control showed very less transcript although both the genes are reported to be expressed ubiquitously in the spikes.

3.1.2.1.4 Transcript evaluation of ΔTa-GSL gene in knock down lines:

Semi quantitative real time PCR was used to evaluate the presence of mRNA transcript in the Δ*Ta-GSL* lines in comparison with the non transgenic control.

RESULTS

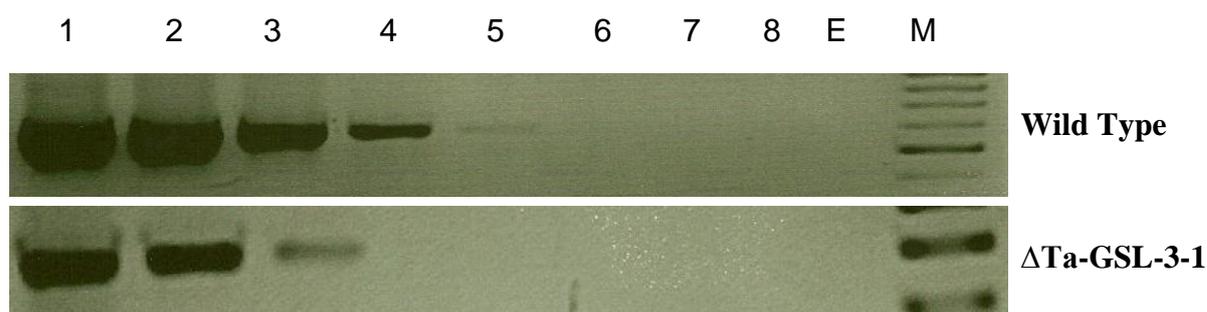


Figure: 3.40 Semi quantitative real time PCR for $\Delta Ta-GSL-3-1$ lines.

Lane1= transcript after 40 cycle, lane2= transcript after 35 cycles....lane8= transcript after 5 cycles, E= empty lane and M=marker.

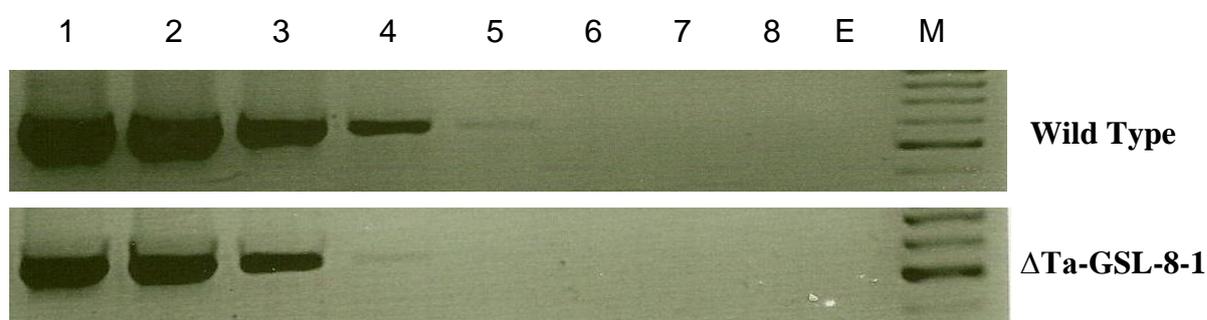


Figure: 3.41 Semi quantitative real time PCR for $\Delta Ta-GSL-8-1$ lines.

Lane1= transcript after 40 cycle, lane2= transcript after 35 cycles....lane8= transcript after 5 cycles, E= empty lane and M=marker.

cDNA was synthesized from RNA isolated from spikes of the knock down lines as well as control and PCR reaction was done with primers designed in a way that the short sequences used in the constructs were the part of amplicate. A total of eight reactions were done for each gene under study. One reaction probe was removed from thermocycler after every five cycles. The results showed that in case of $\Delta Ta-GSL-3-1$ the transcript started appearing at 30th cycle and in case of $\Delta Ta-GSL-8-1$ it appearing after 25th cycle and in case of wild type plants it appeared after 20th cycle. The behaviour of the $\Delta Ta-GSL-3-3$ and $\Delta Ta-GSL-8-3$ was exactly like control line Florida.

3.1.2.1.5 Pathological studies with $\Delta TaGSL$ Knock Downs:

All four knock down lines were tested in the T₁ generation for their reactions to plant pathogen.

FHB (*Fusarium graminearum*) infection studies:

Spikes were inoculated in the middle two spikelets with 200 conidia per spike in 10 μ L water just after fertilization, detailed procedure can be seen in section 2.2.6.2. The infection process was followed until three weeks.

RESULTS

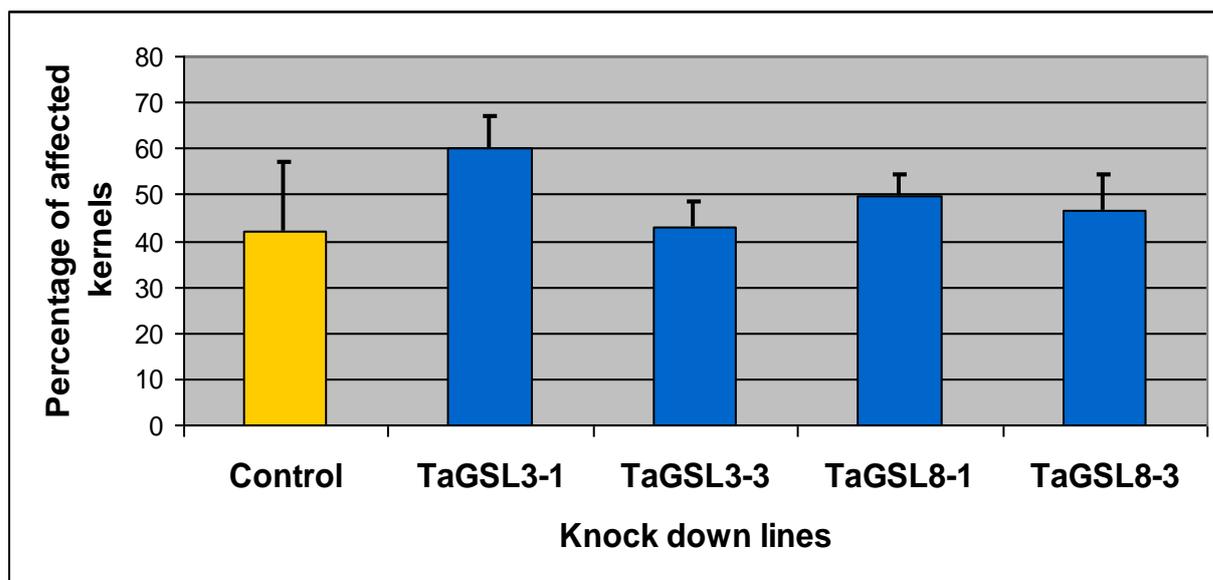


Figure: 3.42 Fusarium Interactions with TaGSL Knock Downs

Figure: 3.42. Shows the percentage of the visually effected kourlns 21 dpi in the non transgenic controls along with knock down lines ($\Delta Ta-GSL3-1$, $\Delta Ta-GSL3-3$, $\Delta Ta-GSL8-1$ and $\Delta Ta-GSL8-3$). The inoculations were made with 200 conidia per spikelet in the middle two spikelets just before fertilization. The experiment was kept at recommended condition for *F. graminearum* development stated in the section 2.2.6.2 of this manuscript

Three weeks after inoculations the data were collected for the number of visually affected kernels per spike. The results showed a detectable change in the number of visually affected kernels in the knock downs as compared to the control line. Knock downs looked more susceptible to the *F. graminearum* compared with the control plants. Non transgenic control line Florida showed an average of 42.05% visually affected kernels. Line *Ta-GSL3-1* looked most susceptible than the rest with an average of 60.14% visually effected kernels. *Ta-GSL8-1*, *Ta-GSL8-3* and *Ta-GSL3-3* got on an average of 49.79%, 46.431% and 46.25% visually affected kernels per spike respectively. It is concluded that knock down genotypes have an impact on the resistance of wheat against at least *F. graminearum* and wheat loses some resistance due to the knock down of both of these genes separately.

RESULTS



Figure: 3.43 the representative experimental examples for FHB on Knock Down and control lines.

The figure 3.43 shows the original experimental example of the infected spikes of knock down lines 9 dpi and 21 dpi. Increased susceptibility of the knock down lines can be seen in the form of more number of affected spikelets per spike as compared to the non transgenic plants while the non inoculated spikes showed no disease symptoms proving that the symptoms are produced by fungi and the difference is susceptibility is due to the knock down of *GSL* gene.

3.1.3 Progeny Segregation among the transgenic lines:

The segregation of the transgenic lines was checked by spraying the young seedlings with herbicide *BASTA* twice, 7 days after germination and 15 after germination at 150 mg/L and 200 mg/L.

Table: 3.5 Segregation among progenies of transgenic lines

Transgenic line	Percent germination	No. of Dead Plants	No. of surviving Plants	Percentage of surviving Plants
Knock Down Lines:				
$\Delta Ta-GSL3-1$	90	18	72	80
$\Delta Ta-GSL3-3$	95	45	50	52.63
$\Delta Ta-GSL8-1$	96	12	84	87.5
$\Delta Ta-GSL8-3$	100	23	77	77

The ratio among surviving and dying plants seemed to be 3:1 except $\Delta Ta-GSL3-3$ which indicates the single locus integration of selection marker gene. The detail is shown in table 3.5.

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3.2 Identification of Genes for Disease Inducible Promoter identification from wheat:

The success of a transformation project largely depends upon the ability of the plant species to successfully regenerate the transgenic plants. The regeneration capacity of the plant species is predominately a genetic character but some times the product of the transgenic gene interferes with plant developmental process during regeneration and transgenic dies at embryoid stage. The solution of such instances is the use of inducible promoters. In the present project constitutive promoter as well as stress inducible promoter was used but the results seemed to have an effect of promoter on regeneration. In the gene silencing experiments very low number of transgenic plants could be regenerated and those too were not complete knock downs. Later it was found out that constitutive expression of gene knock down cassette is toxic for transgenic regeneration. A stress and disease inducible promoter was used in the co-expression of *HarChit* and *HarCho* genes but the number of plants regenerated was comparable to the situation where constitutive promoter was used. The probable explanation of these results is that plant transformation and regeneration is itself a process with a lot of stress and the promoter was already reported to be active 48 hours after biolistic bombardment (Leckband, 1997). Under these circumstances it was decided to find out some genes those are expressed only under disease and the promoter from which can be used in future transformation experiments for disease resistance.

An extensive literature survey was done to find out the genes those are up-regulated upon *F. graminearum* infection. In this process ten genes / ESTs were selected from one Affimatrix chip prepared for transcriptome analysis of barley upon *F. graminearum* infection. Additionally, two ESTs were selected from a publication by Kruger *et al.*, (2002). These genes were reported by them to be up regulated upon *F. graminearum* infection in semi resistant cultivar “Sumai-3”. The selected genes from barley were used to find out the genes with maximum similarity in wheat by running a blast search.

3.2.1 Expression analysis of selected genes in wheat cultivar “Florida”:

The selected genes were checked to be expressed under *F. graminearum* inoculations as well as under mock control in wheat genotype “Florida”. Florida spikes were inoculated with *F. graminearum* and in parallel with water as mock control. These spikes were harvested after different time points (selected time points can be seen in 2.2.7). The infection procedure can be seen in 2.2.6.2. All the infections were done in triplicate and the experiment was repeated thrice. RNA was isolated from the infected and control spikes, RNA of all the three spikes of

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one treatment was pooled and a part of it was used for cDNA synthesis. The rest was stored at -70°C to be used later for Northern Blot analysis.

3.2.1.1 Expression analysis by PCR

The synthesized cDNA was used to run PCR reactions separately for all the twelve genes on standardized protocols. The selected genes and primers used can be seen in table. 2.2.7.

Out of twelve genes / ESTs studied only four looked to be up regulated and the rest either did not express or were equally expressed on mock inoculations and pathogen inoculations. Out of the two ESTs already reported Kruger *et al.*, 2002, one was equally expressed on control and pathogen inoculations and the other one was even absent from DNA in “Florida”.

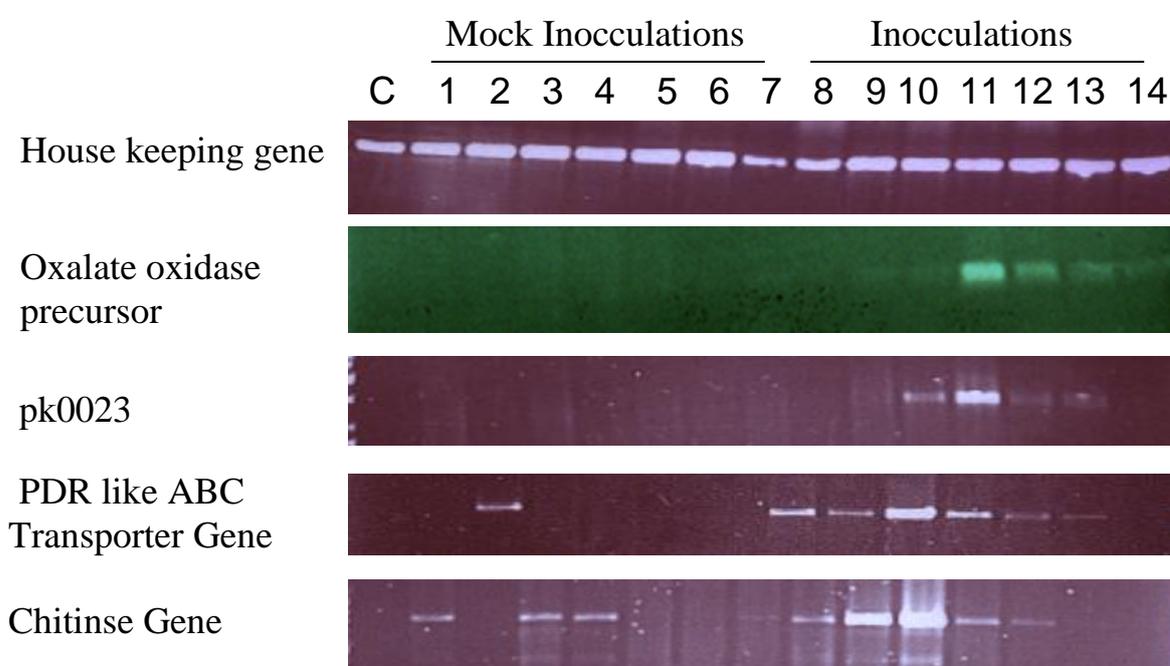


Figure: 3.44 wheat genes up-regulated upon *F. graminearum* infection

Figure 3.44 shows the PCR reaction with gene specific primers of the four genes which are differentially expressed under *F. graminearum* infection.

Lane C is the un-inoculated Florida used as -ve control, lanes 1-7 are inoculations made with 10µL water (Mock control) harvested after 12ha, 24ha, 48ha till 144ha. Lanes 8-14 are inoculations made with 200 conidia in 10µL of water and harvested after 12ha - 144ha post inoculations.

As is shown above, PCR of the house keeping gene 18^S proved normal isolation of RNA and synthesis of cDNA. It also indicates that it is possible to amplify a gene/fragment from c-DNA if the transcript is present in the RNA. In all the four genes out of twelve, the genes / ESTs were off under normal conditions because there was never a band in the lane from untreated control plant. The lanes which carried PCR products from the mock control / water

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control probes they did not show expression in general. In some cases, some times there was expression but the level was very low and there was no consistency. It means either it was some kind of contamination in RNA and cDNA handling or cross contamination during infection process.

The expression was always seen for probes taken from *F. graminearum* inoculated spikes. The expression starts at 12 hai at very low levels. It keeps on increasing and get maximum at 72hai. After getting its peak the expression level goes on decreasing and the minimum level is seen at 144 hai. In case of “PDR like ABC Transporter gene” the maximum expression was seen at 48hai while there seemed no expression of “Chitinase” at 144hai.

3.2.1.2 Expression analysis by Northern Blot:

On the basis of the results taken through PCR the four selected genes were checked for the up regulation of expression upon *F. graminearum* inoculation. Small fragments were amplified from all the four genes and cloned in Topo-Ta cloning vector. The fragments were separated from the plasmids and eluted out of the agarose gel. These fragments were then radio actively labelled with P³² and used in the Northern Blot analysis for all the genes under study. The process of Northern Blot analysis is stated in section 2.2.5.6. A total of four RNA gels were prepared for RNA harvested from respective treatments. RNA was taken from the same test tube for a treatment on all the four gels.

RESULTS

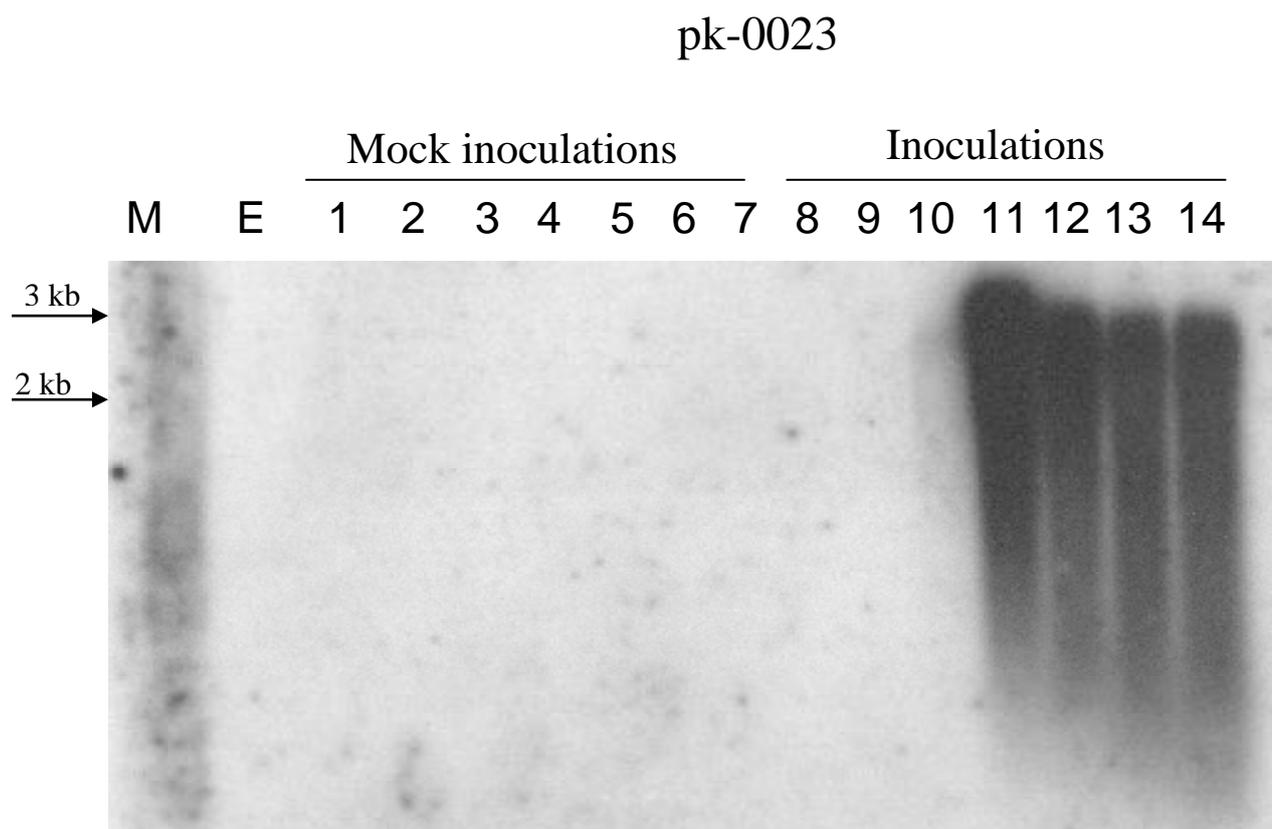


Figure: 3.45a Northern Blot analysis for pk0023 gene.

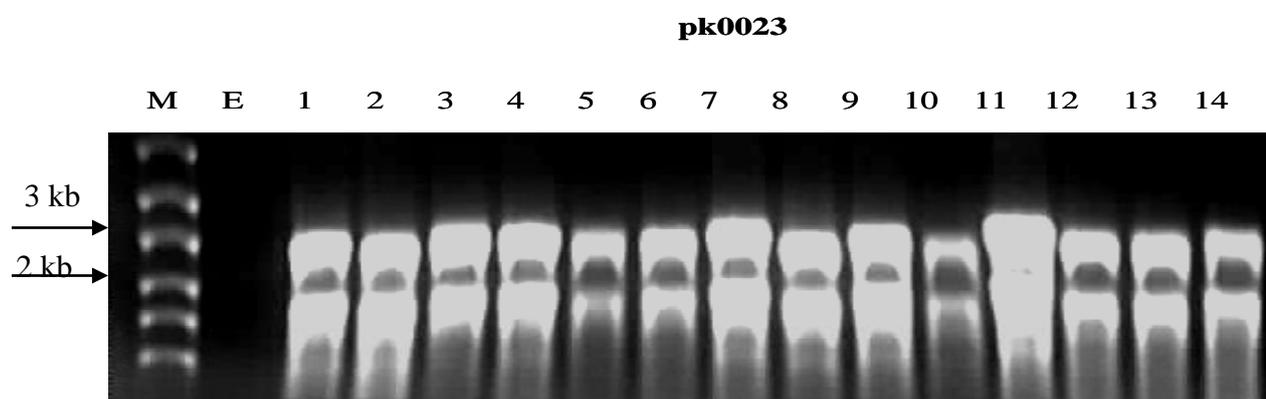


Figure: 3.45b control gel for Northern Blot analysis of pk0023

Freshly isolated RNA from mock inoculated and inoculated probes were run on 1% degeneration agarose gell and RNA was transferred to the nylon membrane by capillary method and cross linked. *Pk0023* specific 400 bp long single stranded radio actively labelled DNA probe was later used to hybridize RNA specific to pk0023 gene. The results were seen on the X-rays film.

M= High range RNA marker, C= Control plant with out inoculation, 1-7 mock inoculated probes harvested 12hai, 24hai, 48hai..and 144hai. 8-14 *F. graminearum* inoculated probes.

RESULTS

The results showed the same trend as it was shown in the last section where the expression was studied by PCR. On three out of four selected genes there was no indication of any expression for mock controls. On *F. graminearum* inoculated probes the expression seems to start atleast 48 hai and the maximum expression is found at 72 hai and 96 hai which keeps on decreasing till 144 hai.

In terms of the quantity of the transcript pK0023 seemed to express abundantly. Slight expression started at 48 hai and increased abruptly at 72hai and then started decreasing after that.

PDR like ABC Transporter gene

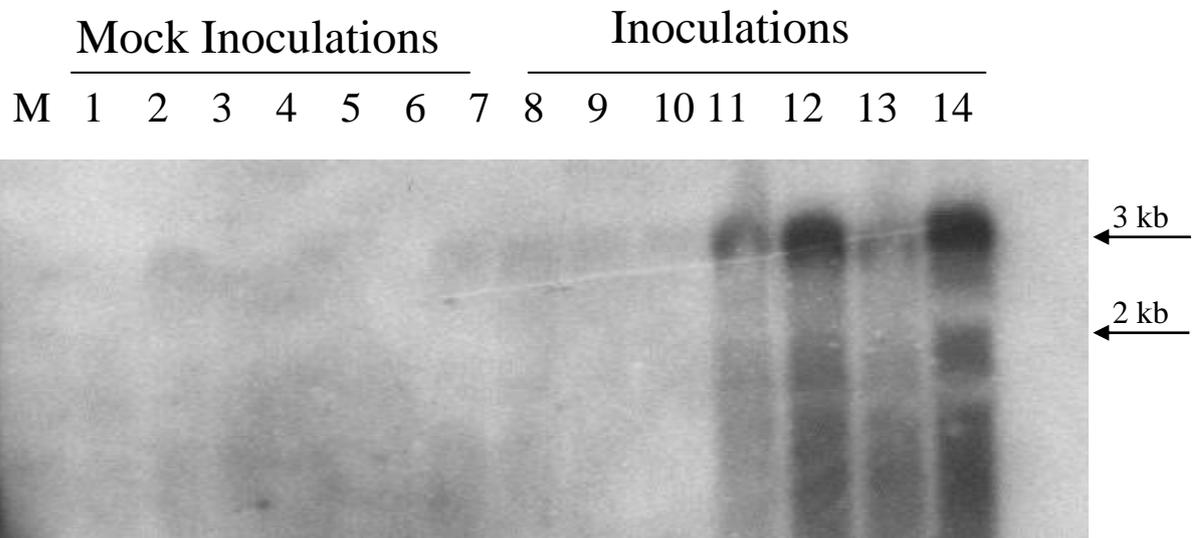


Figure:3.46a Northern Blot analysis for PDR like ABC Transporter gene

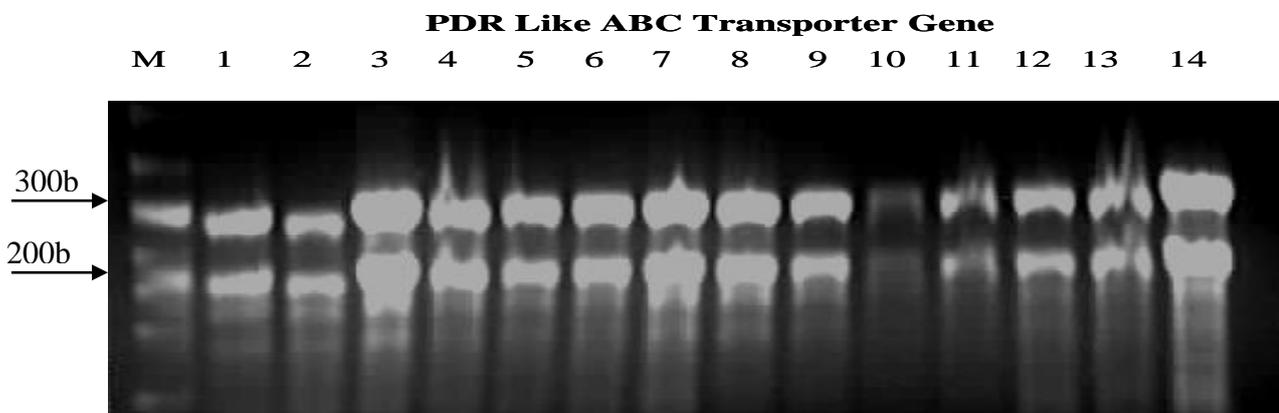


Figure 3.56b control Gel for4 Northern Blot analysis of PDR like ABC Transporter gene

Freshly isolated RNA from mock inoculated and inoculated probes were run on 1% degeneration agarose gell and RNA was transferred to the nylon membrane by capillary method and cross linked. “PDR like ABC Transporter Gene” specific 400 bp

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long single stranded radio actively labelled DNA probe was later used to hybridize RNA specific to “PDR Like ABC Transporter Gene”. The results were seen on the X-rays film.

M= High range RNA marker, C= Control plant with out inoculation, 1-7 mock inoculated probes harvested 12hai, 24hai, 48hai .and 144hai. 8-14 *F. graminearum* inoculated probes.

The expression of PDR like ABC transporter gene was also differential for inoculated and mock inoculated control but the expression level was seemingly low as compared to *pk0023*. At the same time there seemed very little spots in the mock control lanes on the film which indicated little expression at mock control as well.

The expression of “*Oxalate oxidase precursor gene*” was absolutely differential and there were no signs of expression on non inoculated control as well as on the mock control. Expression started at 72 hai and went upto 144 hai but the level of expression was very low compared to other genes.

In case of “chitinase gene” the story was a little bit different; here we can see the differential expression of the gene, expression gets very high at 72 hai and 96 hai and then decreases a bit going to 144 hai with a little change, the expression level is high as well. At the same time we can also see some expression in all the lanes with mock control although this expression is very low as compared to inoculated ones.

Oxalate oxidase precursor

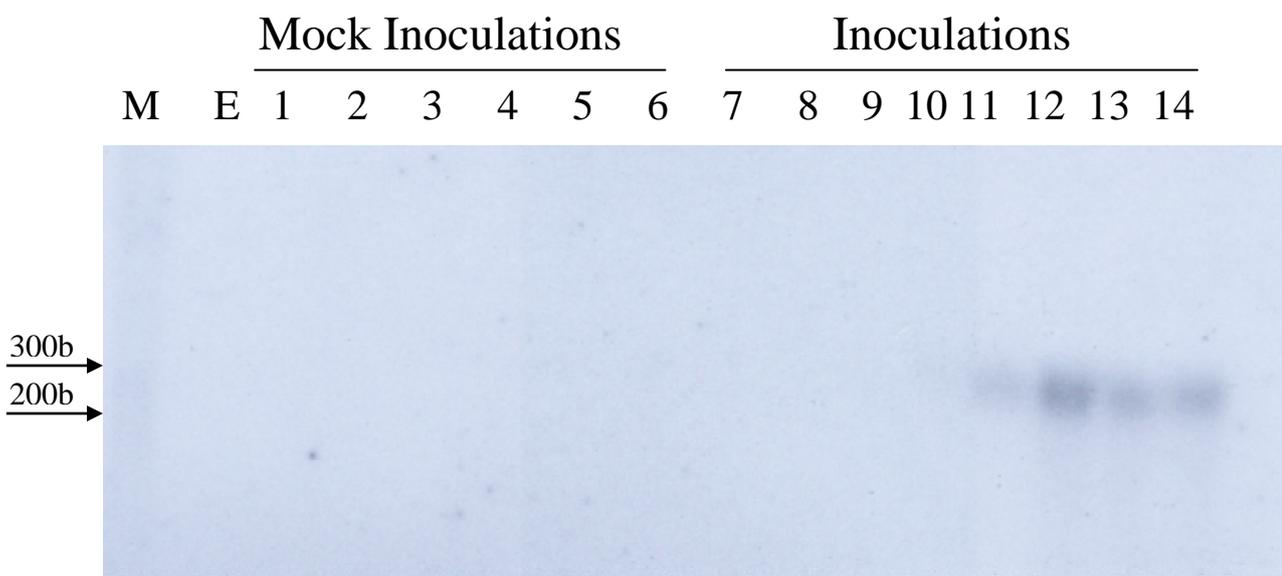


Figure: 3.47a Northern Blot analysis for Oxalate Oxidase precursor gene.

RESULTS

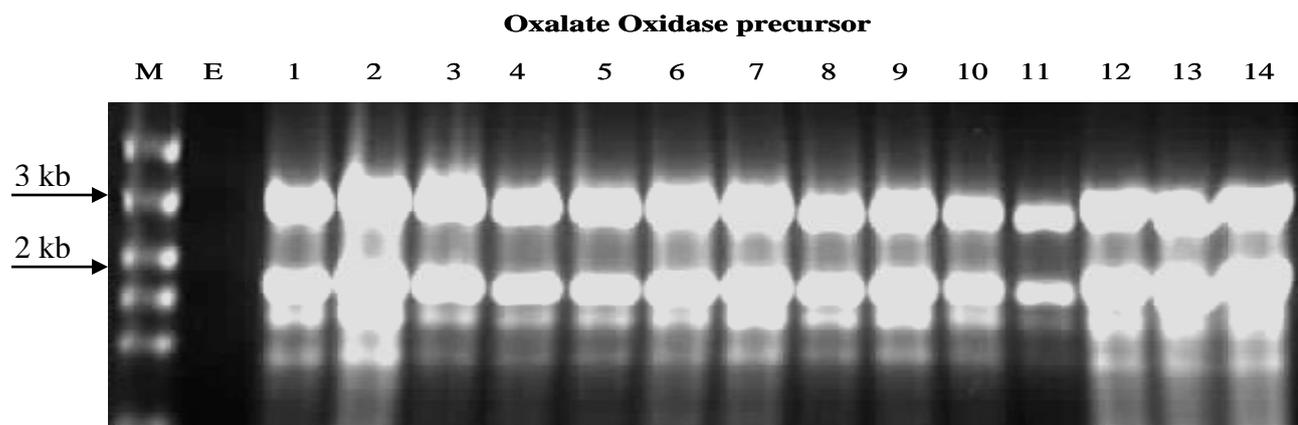


Figure: 3.47b control gel for Northern Blot analysis for Oxalate Oxidase precursor gene.

Freshly isolated RNA from mock inoculated and inoculated probes were run on 1% degeneration agarose gell and RNA was transferred to the nylon membrane by capillary method and cross linked. "Oxalate oxidase precursor" specific 400 bp long single stranded radioactively labelled DNA probe was later used to hybridize RNA specific to "Oxalate oxidase precursor". The results were seen on the X-rays film.

M= High range RNA marker, C= Control plant with out inoculation, 1-7 mock inoculated probes harvested 12hai, 24hai, 48hai .and 144hai. 8-14 *F. graminearum* inoculated probes.

Ta-Chitinase

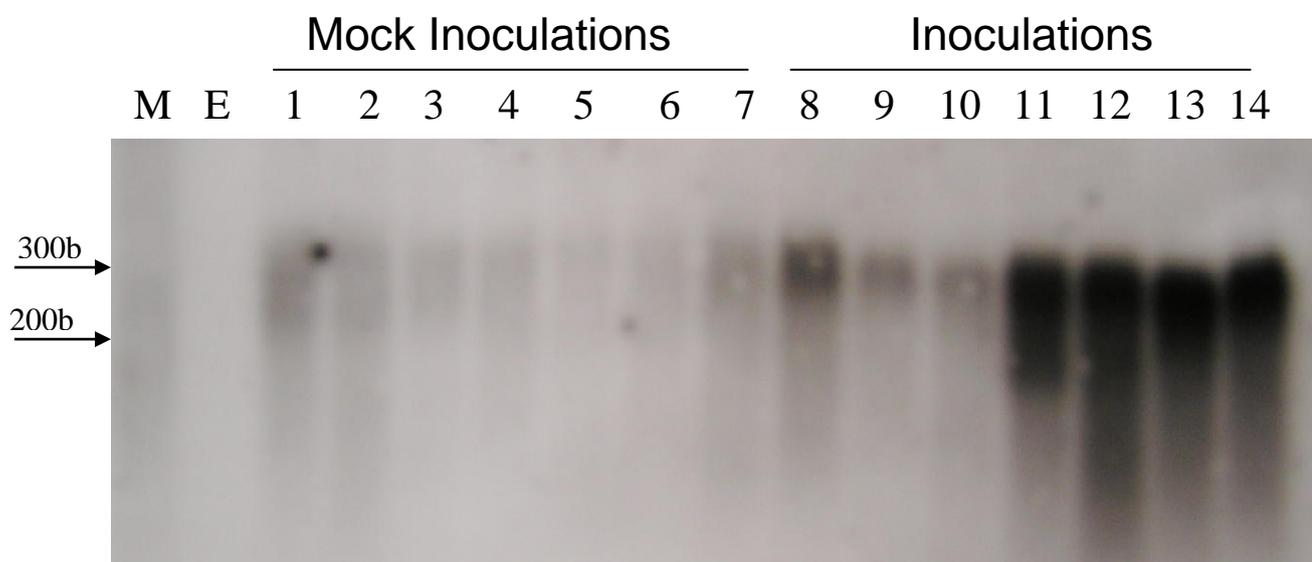


Figure: 3.48a Northern Blot analysis for chitinase gene.

RESULTS

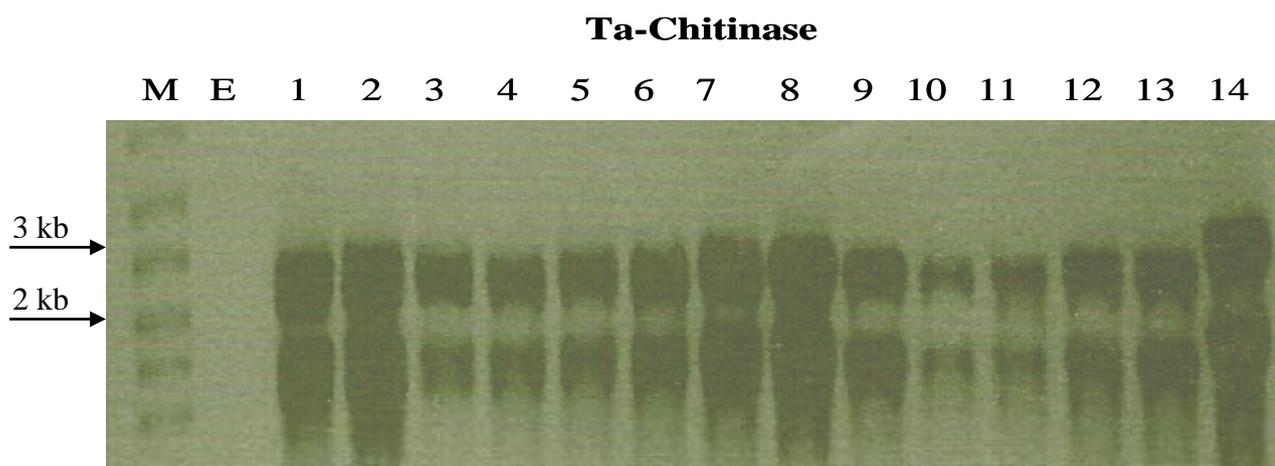


Figure: 3.48b Control gel for Northern Blot analysis of Ta-chitinase gene.

Freshly isolated RNA from mock inoculated and inoculated probes were run on 1% degeneration agarose gell and RNA was transferred to the nylon membrane by capillary method and cross linked. “*Ta-Chitinase*” specific 400 bp long single stranded radio actively labelled DNA probe was later used to hybridize RNA specific to “*Ta-Chitinase*”. The results were seen on the X-rays film.

M= High range RNA marker, C= Control plant with out inoculation, 1-7 mock inoculated probes harvested 12hai, 24hai, 48hai .and 144hai. 8-14 *F. graminearum* inoculated probes.

All the four genes studied above seem suitable candidates for finding promoters as the promoters which are regulating their expression are sensitive for *F. graminearum* infection. “pk0023” is a best candidate as it not only expresses differentially but the expression level is very high as well. The differential expression level of “Chitinase” as well as of “PDR like ABC transporter gene” is high as well but there seems some expression at mock inoculations which may weaken their case. “Oxalate Oxidase Precursor gene” is although differentially expressed and there is no expression on mock inoculation but the expression level is too low.

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Table: 3.6. Performance of over expression lines to FHB and PM

Transgenic Line	Change in resistance to FHB	Change in resistance to PM
Over expression lines (under the Ubi-1 promoter)		
I.A-1	Not evaluated	++
I.A-2	Not evaluated	Not evaluated
I.A-3	++	+++
I.A-4	Not evaluated	++
I.A-5	Not evaluated	Not evaluated
I.A-6	+	++
I.A-17	Not evaluated	Not evaluated
I.A-18	+	+++
Over expression lines (under the <i>Vst-1</i> promoter)		
I.A-7	Not evaluated	+
I.A-8	++	-
I.A-9	Not evaluated	--
I.A-10	Not evaluated	-
I.A-11	+	++++
I.A-12	++	++

Note: ++++= more 69% increase in resistance, +++= 50-69% increase in resistance, ++= 30-49% increase in resistance, += 10-29% increase in resistance, -= 10% decrease to 10% increase in resistance, --= more than 10% decrease in resistance.

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4. DISCUSSIONS:

Wheat along with maize and rice is one of the most important food crops in the world. It is most vulnerable to diseases. The goal of the present work was to try and find out the ways to create solutions for tackling these diseases at plant level. This goal was achieved by dividing the project into three parts, in the first instance two antifungal genes *HarChit* and *HarCho* producing antifungal enzymes from *Trichoderma harzianum* were cloned separately under constitutive *Ubiquitin* promoter and stress inducible *Vst1* promoter. These genes were separately co-transformed into wheat and their expression was taken constitutively as well as under stress. For the transformation of wheat already reported protocols were used. In the second part three selected members of the *Ta-GSL* family were tried to knock down by PTGS using different *RNAi* constructs. Out of the three genes it was never possible to get a transgenic for *Ta-GSL10* while 2 plants/lines were found each for *Ta-GSL8* and *Ta-GSL3* but only when the siRNA forming fragments of around 150 bp were used in *RNAi* constructs. No plant was found when siRNA forming fragments of 230 bp were used. The individual transgenics with *RNAi* constructs showed a reduction in the transcription of the concerned genes. As a third part of this project it was tried to find out the genes those are differentially expressed under disease attack. The purpose was to use these genes for the identification of their promoter in future that can be used as disease inducible promoter. For this purpose four genes were found out those are expressed only on *F. graminearum*.

Pathological studies with the transgenic lines showed a decrease in the damage caused by PM fungus as well as by *F. graminearum*. When both the genes (*HarChit* and *HarCho*) were expressed constitutively a decrease of upto 60% and when the genes were induced by fungal inoculations a decrease of upto 75% was recorded in the size of PM colonies. The decrease in the spread of *F. graminearum* was seen upto 41.03% decrease in visually effected kernels when the genes were constitutively expressed and a decrease of upto 43.77% when the genes were induced by fungal attack. Interesting, for both the fungi every line behaved differently. One line which was good against PM was not always good against FHB. The *RNAi* transgenic lines were checked against *F. graminearum* which showed upto 43% increase in visually affected kernels for $\Delta Ta-GSL3$ and up to 18.4% increase for $\Delta Ta-GSL8$ lines. It gives a conclusion that both of these genes have an influence in the resistance of wheat against at least *F. graminearum*.

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4.1 Wheat Transformation:

Wheat transformation started in the end of 1980's and the protocols for transformation has been improving since then. Traditionally wheat is transformed by biolistic bombardment of the plasmid DNA onto the *explant*. The first wheat transgenic plant was recovered in 1992. After that; work has been done on the improvement of parameters involved in the wheat transformation process. Wheat embryogenic callus, leaves, roots, stem portions, panicle, mature and immature embryos has been tried as *explant* sources for transformation. Different vacuum pressures, metals of the particles *e.g* gold or tungsten and the sizes of particles, selection agents as well as the different regimes of tissue culture media have been used. The most widely and successfully used protocols now; include immature embryos as explants source, gold as metal and 0.8-1.4 μm^2 the size of the particle, *kanamycin* or *BASTA* as agents for selection while modified MS media with varying levels of 2,4-D (Vasil *et al.* 1992 and 1993, Becker *et al.* 1994, Takumi and Shamida, 1996, Leckband and Loerz, 1998, Jones, 2005). Wheat transformation via *A. tumefaciens* was also started in parallel in 1990's and was reported in 1997. The work is still continued over it considering the factors like *explant* sources, strains of the *A. tumefaciens* and virulence factors along with the wheat genotypes to be transformed. Wheat transformation using *A. tumefaciens* is still in its infancy and is not being used in routine wheat transformation experiments. Wheat transformation via biolistic bombardment is being reported in many important experiments of developmental and applied biology of wheat (Sivamani *et al.*, 2000, Oldach *et al.*, 2002, Anand *et al.*, 2003, Becker *et al.*, 2007 and Shin *et al.*, 2008). In all these studies wheat transformation frequency has been reported nearly 1% of the total explant tissues tried. There is only one report where Pellegrineshi *et al.*, 2002 reported the transformation frequency of more than 60% in 8 sister lines of genotype 'Bobwhite'. In some experiments where the gene silencing was tried through stable genetic transformation, the situation seems significantly worsened. Yan *et al.*, 2004, Loukoianove *et al.*, 2005 and Regina *et al.*, 2005 tried gene silencing in wheat successfully but in first two cases only one plant was found and two transgenic plants were found in the third report. In addition it was not possible to show the phenotypic stability in the coming generation. While Folckk *et al.*, 2004, Travella *et al.*, 2006 and Humanes *et al.*, 2008 showed more than one plant transformed with RNAi constructs and the resultant lines were stable genotypically as well as phenotypically.

It was decided to use biolistic bombardment technique for wheat transformation in the present project. The results achieved in this project showed a mixed trend when compared to other

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projects done in the past. In the over expression experiments the transformation frequency was slightly less than 1% while in the gene silencing experiments only four plants could be recovered for two different RNAi constructs the details for whom will be discussed later. The transformation experiments were done over a time span of one year but the transformants were found between November and April, this was what found by Brettschneider *et al.*, 1997 during Maize transformation under Hamburg conditions. It indicates that wheat and maize need some specific physiological conditions which are produced in these plants only during the above mentioned time period. The most probable situation is the day/night length because the rest of the conditions are mostly controlled.

4.1.1 Co-expression of *HarChit* and *HarCho*:

The defence response genes function in a variety of ways to inhibit fungal infection and expression of these genes in transgenic plants has been shown to enhance fungal resistance (Muehlbauer and Bushnell, 2003). Wheat like other plants has an innate defence response against the fungal pathogens that involves the induction of PR genes at the rear end. These PR genes defend by attacking different organs of the pathogen. For example chitinases and 1, 3- β -glucanases hydrolyse the fungal cell wall by targetting fungal cell wall chitin (poly-GlcNAc) and 1, 3- β -Glucan as substrate. There have been reported many chitinase genes in wheat and their antifungal activity is established. Botha *et al.*, 1998 reported 7 constitutively expressed and 3 pathogen induced chitinase isoforms in wheat. Singh *et al.*, 2007 isolated 33kD chitinase from wheat and expressed it in *e.coli* to prove its antifungal role *invitro*.

Due to the interest generated from their lytic action against fungal cell wall, chitinase genes have been isolated, sequenced and cloned from bacteria, yeast, plants and filamentous fungi (Jones *et al.*, 1986; Watanabe *et al.*, 1990; 1992; Kuranda and Robbins, 1991; Collinge *et al.*, 1993; Yanai *et al.*, 1992 and Blaisean and Lafay, 1992).

Chitosanase is an enzyme similar to chitinase, capable of hydrolyzing the β -1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated fungal cell wall polymer. Chitosanases have the potential of slowing or preventing fungal infection by degrading the structural chitosan (poly-GlcN) found in the cell wall of many fungi (Hendrix and Stewart, 2002). Glucose amine oligomers, released from fungal cell walls after hydrolysis with chitinase or chitosanase, are elicitors of plant defence response (Lee *et al.*, 1999 and Vander *et al.*, 1998). The response elicited by these molecules depends on the length and degree of acetylation of the oligomers released (Vander *et al.*, 1998). Long oligomers or intact fungal cell walls will cause little or no reaction. Oligomers that are

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relatively short (e.g. products of chitosanase hydrolysis) are active elicitors of plant defence systems.

T. harzianum, a soil-borne fungus known to be a control agent of fungal plant pathogens (Papavizas, 1985), is one of the fungi that produce degrading enzymes which destroy key cell wall structural polymers of fungal pathogens (Hendrix and Stewart, 2002). The purified enzymes from *T. harzianum* are strong inhibitors of many important plant pathogens and are also able to lyse not only the 'soft' structures of the hyphal tip but also the 'hard' chitin wall of mature hyphae, conidia, chlamydospores and sclerotia (Lorito *et al.*, 1998). They are substantially more chitinolytic and glucanolytic than the enzymes from other known sources (*i.e.* upto 100 times more active than the corresponding plant enzymes and effective on a much wider range of pathogens) (Lorito *et al.*, 1994; Lorito *et al.*, 1996). They are also non toxic to plants even at high concentration (Carsolio *et al.*, 1998). Furthermore, the anti-fungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used together or in combination with plant PR-proteins, commercial fungicides, cell membrane-affecting toxins or biocontrol bacteria (Lorito *et al.*, 1998; Steyaert *et al.*, 2004). Co-expression of chitinase and chitosanase genes from *T.harzianum* could therefore result in a synergistic enhancement of anti-fungal activity of wheat.

Constitutive promoters have been used in maximum transgenic experiments in all the plants. The use of CaMV promoter and Ubiquitin promoter is very traditional in dicots and monocots respectively. But this choice is not absolute. In some experiments specially related to disease resistance the expressed protein is not required all the time or it is needed only in some specific parts of the plant. When it is expressed constitutively, there comes extensive cellular reprogramming of defence components in the parts of the plants which are not infected. This may give some times rise to plants which are resistant to diseases but weak in health and resulting productivity (Gurr and Rushton, 2005). For example in Arabidopsis NPR1 over expression using CaMV 35S promoter brought broad spectrum resistance with normal phenotype while in maize same was done using *Ubiquitin* promoter and it gave rise to diseased genotype with out infection (Cao *et al.*, 1998; Piererse and Loon, 2004). Keeping in view these reasons and the fact that chitinase and chitosanase enzymes of the *T. harzianum* are highly antifungal it was decided to co-express *HarChit* and *HarCho* genes under constitutive as well as under inducible promoter. The reason was that success stories of the constitutive promoters in disease resistance have already been published and at the same time there was a chance of failure. We used *Vst-1* promoter from grape wine as a stress inducible promoter that is also induced under disease in wheat (Leckband and Loerz, 1997). Ubiquitin

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promoter from Maize was used as constitutive promoter for the co-expression of *HarChit* and *HarCho*.

HarChit and *HarCho* genes from *Trichoderma harzianum* were cloned separately under constitutive *Ubiquitin-1* promoter from maize and stress inducible *Vst1* promoter from grape wine (*Vitis vinifera*). These were then co-expressed separately in wheat using the modified transformation protocols reported by Becker *et al.*, (1994), Brettschneider *et al.*, (1997) and Oldach *et al.*, (2001).

4.1.2 Co-expression of *HarChit* and *HarCho* under Constitutive Promoter:

A total of 34 plants were found resistant to selection agent *BASTA* at the completion of transformation experiments. These plants were then proved for the presence of *HarChit* and *HarCho* genes by Southern Blot analysis. The results of the Southern Blot analysis proved that only 8 (23.53% of total transgenics plants) plants had the constructs of interest. This result looks different from Mathews *et al.*, (2001) who showed upto 70% co-expression of genes of interest and the selection marker gene in Barley and Bliffeld *et al.*, (1999) who got 100% co-integration in wheat but exactly like Pellegrineschi *et al.*, (2003) who found only 25% transgenic plants with the gene of interest out of 100% containing selection marker gene. The cause of this big difference was not studied as it was not in scope of this project. Out of 8 plants which were having the genes of interest 6 showed the co-integration of *pUbi-HarChit* and *pUbi-HarCho* while one plant had only *pUbiHarChit* and the other only *pUbiHarCho*. The transformation frequency for the selection marker gene was 1.08% which is in accordance with most of the reports for transformation as is reported by Becker *et al.*, (1994), Bliffeld *et al.*, (1999), Oldach *et al.*, (2002). The frequency calculated for the genes of interest was only 0.26%. This frequency is better than the transformation frequency found by Fettig and Hess, (1999) who found 8 transgenic plants from genotype 'Combi' and 'Hanno' at a frequency of 0.02% and 0.01% respectively. Southern Blot analysis results showed single copy and multicopy integration for *pUbi-HarChit* (1-3 copy integration) and *pUbi-HarCho* (1-7 copy integration). Ayoo, (2008) presented single copy integration for *HarChit* and multicopy integration for *HarCho* in Sorgham. All the plants looked normal phenotypically except *IA-1* (co-integration of both the genes) and *IA-2* (integration of *HarChit* only). These plants were short statured with stunted growth. *IA-1* was partially fertile while *IA-2* had male sterility. The progeny of *IA-2* was taken by cross fertilisation with wild type wheat pollens. Plants of the *IA-2* were normal in T₁ generation while *IA-1* plants showed normal growth initially but later turned stunted with late maturity. This is possibly due to somaclonal

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variation affects stated by Lazzeri and Shewry, (1993) and are expected to fade away after a few generations. All the T₀ and T₁ plants were checked for the expression of both genes by Northern Blot analysis and the genes were co-expressed in 4 out of six lines. Lines *I.A-1* and *I.A-5* which showed co-integration of *HarChit* and *HarCho* but the expression was present for *HarCho* only in *I.A-1* and there was no expression of *HarChit* or *HarCho* in *I.A-5*. The lines *I.A-2* and *I.A-17* which showed the integration of only *HarChit* and *HarCho* respectively, showed the expression of them in T₀ and T₁ generation plants. The non expression of the *HarChit* and *HarCho* in *I.A-5* and *HarChit* in *I.A-1* can be attributed to biolistic bombardment experiments where it happens from time to time.

4.1.3 Co-expression of *HarChit* and *HarCho* under Inducible Promoter:

31 *BASTA* resistant plants were recovered in the transformation experiments done for the co-integration of *pVst-HarChit* and *pVst-HarCho* constructs. The analysis for the genes of interest showed 6 plants out of 31 that contained gene/genes of interest. Out of these 6 plants one plant '*I.A-8*' had only *pVst-HarCho*. For the expression analysis of *HarChit* and *HarCho* on stress an rt-PCR was done and found out that the expression analysis followed the same trend as Southern Blot analysis. Out of six plants 5 showed the co-expression of both genes while the 6th that contained only *HarCho* showed the transcript only for *HarCho* only. When the transformation frequency was calculated, it was 0.87 for the selection marker gene and 0.17 for the genes of interest. *PVst-HarChit* showed 1-3 copy integration and *pVst-HarCho* showed 2-9 copy integrations in wheat genome.

In general, the transformation of all the members of poacea family is a tough task and the protocol establishment via *A. tumefaciens* transformation as well as biolistic bombardment is under continuous improvement. There have now been some reports on wheat transformation via biolistic bombardment but the transformation frequency still is very low. Vasil *et al.*, (1992) and (1993), Becker *et al.* (1994) showed wheat transformation with marker genes and evaluated various transformation improvement parameters but the transformation frequency was still around 1-2%. Chugh and Khurana, 2003 reported 4% transformation frequency. Pellegrineschi *et al.*, 2001 showed the transformation frequency of 60-89% in some sister lines of the 'Bobwhite' but these results were never repeated in other labs worldwide. Some recent reports on wheat transformation with the genes of interest also showed low transformation frequencies 0.02% by Fettig and Hess, (1999), 0.7% Bliffeld *et al.*, (1999), and 0.3-2.6% by Oldach *et al.*, (2001).

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4.1.3.1 Transgenes Progeny Segregation

It was possible to advance all the plants into the next generation by self pollination except one that was male sterile and cross pollination was done to have its T₀ seeds. The self pollinated seed was harvested from the spikes of the primary transformants and T₁ generation was obtained by sowing this seed in the green house and transgenic plants were selected by *BASTA* sprays. Single locus integration was expected as per previous reports but out of 5 progenies (*I.A-3*, *I.A-4*, *I.A-5*, *I.A-6* and *I.A-18*) of *pUbi-HarChit + pUbi-HarCho* checked one progeny (*I.A-18*) showed entirely the reversed results with 1:3 ratio of surviving to dead plants. In the transformants with *pVst-HarChit* and *pVst-HarCho* two progenies (*I.A-8*, *I.A-12*) showed the single locus integration and 3:1 ratio of the herbicide tolerant to susceptible was seen. One progeny (*I.A-10*) died completely on *BASTA* spray but the dying plants were tested positive for *pVst-HarChit* and *pVst-HarCho* integration and expression. Three progenies (*I.A-7*, *I.A-9* and *I.A-11*) showed nearly reversed ratios than the expected *i.e.* 1:3 instead of 3:1. When the surviving plants from the abnormally segregating progenies were checked for the integration of genes of interest, they showed normal integration pattern and expression like in T₀ generation (the exact data for herbicide resistance to susceptibility can be seen in table 3.2).

This kind of results with transgene silencing has already been shown by many researchers worldwide. Recently Li *et al.*, 2005 and Anend *et al.*, 2003 studied the transgene silencing of wheat in detail. In the experiment of Anend *et al.*, 2003, 20 out of 24 wheat transgenic plants transformed with rice chitinase and glucanase genes with bar as marker gene showed complete gene silencing in T₁ generation and onwards. The rest of the plants showed stable integration and expression of all the three genes till 4 studied generations. Their studies found out extensive methylation of CCGG sequences of the transgenes. Methylation was observed as a random and progressive phenomenon that can even vary in different parts of the plant. They found out by comparing their results with their already presented data that methylation is also specific to promoters used and the plant species transformed. 35S promoter is more prone to methylation than *Ubi-1* promoter of maize same is the case with wheat compared to other cereals. Due to the biolistic bombardment wheat genome has to re-arrange itself with huge genome size and in the process methylation activity is increased. Transgene silencing is also attributed to the high copy number as well as the site of integration. It is interesting here that all the plants in T₀ generation were selected through herbicide resistance created by active selection marker gene. Probably this marker gene went silenced selectively at the end of T₀ generation or start of T₁ generation at embryo formation stage and the resultant plants were

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susceptible to herbicide inspite of having the selection marker gene. The situation where all the plants died the transgene silencing occurred may be at some stage of T₀ generation by increased methylation. The progenies which showed normal single locus integration and showed 3:1 ratio for herbicide resistance to susceptibility did not seem to have any problem transgene silencing.

Another observation in our experiments was the high copy number integration of genes of interest in the transgenic plants where selection marker gene went silent. Here, this can be speculated that may be this silencing was due to the methylation of transgene although the copy number for marker gene integration was not noted. This can be explained on the basis of results presented by Chen *et al.*, 1999 and Anend *et al.*, 2003 where they showed *Ubi-1* promoter is less prone to methylation than CaMV 35S promoter and methylation is increased by high copy number integration into wheat genome. In our experiments genes of interest were under the control of *Ubi-1* promoter and selection marker genes were under 35S promoter so may be it was this promoter which became the reason of methylation on high copy number integration into wheat genome.

4.1.4 Role of *HarChit* and *HarCho* Co-expression in Wheat Disease Resistance:

Wheat has been seen as problem crop in terms of fungal diseases. The diversity of disease causing fungi and the high mutation rates among these fungi has made the problem multi directional. Traditional breeding techniques have been enabling the plant breeders to develop somewhat resistant cultivars. In spite of continuous efforts this problem has always been there. The development of the molecular biology enables us to study the components of natural plant defence in living organisms and make the food crops like wheat resistant against pathogens. In the present project two antifungal genes *HarChit* and *HarCho* from *T. harzianum* (mycoparasitic fungi) were introduced into wheat and their effect against two wheat pathogens were tested.

4.1.4.1 Role of *HarChit* and *HarCho* co-expression against powdery mildew of wheat:

For the evaluation of *HarChit* and/or *HarCho* overexpression lines against powdery mildew of wheat detached leaf segments from young seedlings were used for inoculation with powdery mildew causal organism *Erysiphe graminis* f.sp. *tritici*. This kind of methods was already used in wheat by Oldach *et al.*, (2001) and Girgi *et al.*, (2006) in pearl millet.

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4.1.4.1.1 Test of Transgenics under Constitutive Promoter:

A total of four lines having *HarChit* and *HarCho*, one line with *HarCho* and one transgenic control with *gus* gene under constitutive *Ubi-1* promoter were evaluated against non transgenic control line Florida. Powdery mildew symptoms started to appear on the wheat segments 5-6 days after inoculation with *E. graminis*. Visual observations were taken on the number of developing colonies 9 dpi and colony size 21 dpi. The results showed a decrease in the number of developing colonies on four lines tested while one line showed little more number of developing colonies compared to controls. Transgenic control line having *Ubi-gus* gene construct showed around the same number of colonies per segment of leaf as non transgenic control. Transgenic lines showed approximately a decrease of 12-20% in the number of developing colonies per leaf disk (details can be seen in figure 3.18). After 9 dpi the difference in the size of the colonies for transgenic lines and controls was not visibly noticeable. 21dpi the size of the fungal colonies was visibly different. On an average the fungal colonies on transgenic plants were 40-60% smaller than the colonies on transgenic and non transgenic controls. The colonies at the negative control lines were looking healthier with a lot of powdery mycelium while fungal colonies on transgenic leaves were mostly dead with very less powdery mycelium and the general appearance was brown and smaller colonies. Moreover a little more yellowishness was also observed in transgenic plants compared to transgenics which may be an indication of accelerated Programmed cell death in the fight against fungus. (The details can be seen in figures 3.19-3.22)

4.1.4.1.2 Test of Transgenics under Inducible Promoter:

The behaviour of the T₁ progenies containing *HarChit* and *HarCho* genes under stress inducible gene was almost the same as shown by transgenic progenies containing *HarChit* and *HarCho* under constitutive promoter (above paragraph). Out of the six T₁ progenies (*I.A-7-I.A-12*) tested with *E. graminis* one progeny (*I.A-9*) showed 14% more number of colonies than non the control lines while the rest showed 8-29% less colonies than the controls. Progeny *I.A-8* had only *HarCho* but the resistance response of it against the development of PM colonies was at par with other progenies having both the genes of interest. The size and health of the *E. graminis* colonies developing on the leaf disks of transgenics with the gene/genes of interest and control lines showed visibly smaller colonies with less powdery material while the leaf disks of the control lines showed visibly larger colonies with a lot of white powdery material. Out of the six lines tested, three lines (*I.A-8, I.A-9* and *I.A-10*) showed the same or larger size of colonies than the size of colonies on control lines. Three lines (*I.A-7, I.A-11* and *I.A-12*)

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showed a reduction of 26, 75 and 36% in the colony size respectively compared to the control lines. The colonies on the controls were having more powdery material and were healthier than those found on the lines with over expressed *HaChit* and *HarCho*.

Various defence response genes have been used in wheat transformation for powdery mildew resistance. Oldach *et al.* (2001) observed a reduction of 32-40% in the number of developing *E. graminis* colonies on wheat transgenic line overexpressing Barley class-II chitinase and *Ag-Afp* protein from *Aspergillus giganteus*. There was no reduction in the fungal establishment when barley type-I RIP was overexpressed. Bieri *et al.* (2000) showed no reduction to less reduction in wheat susceptibility to *E. graminis* by high overexpression of RIP. Bieri *et al.*, (2003) overexpressed barley seed antifungal proteins in wheat and checked the effect separately of alone chitinase, β -1,3-glucanase, RIP and Barnase as well as in combinations. RIP transgenics showed maximum reduction in powdery mildew susceptibility while chitinase and β -1,3-glucanase combination showed different levels of increase or decrease in susceptibility. A combination of three antifungal genes *i.e.* chitinase, RIP and β -1,3-glucanase produced by crossing did never showed reduction of susceptibility better than the best parent. These results are in agreement with the results found in the present project. The increase in the susceptibility of the lines *IA-8* and *IA-9* indicate that it is not necessary to quantitatively increase the anti-fungal proteins to increase resistance against powdery mildew disease rather a basal provision of anti-fungal proteins either produced constitutively or induced helps to increase disease resistance. Additionally, foreign antifungal genes have to interact with the endogenous defence response genes/proteins to produce disease resistant genotypes (Bieri *et al.* 2003). Some times this interaction does not bear results as is seen for lines *IA-8* and *IA-9* and seen by Bieri, *et al.*, (2003).

4.1.4.2. Role of *HarChit* and *HarCho* co-expression against Fusarium head blight of wheat:

Transgenic approaches to combat FHB have been reviewed recently by Dahleen, *et al.* (2001). Various degrees of resistance against FHB may be achieved by introducing *in planta* heterologous genes encoding anti-Fusarium proteins. A variety of anti-fungal genes have been isolated and some of their products have been shown to have anti-fungal activity *in vitro* and *in planta*. Anti-fungal genes *HarChit* and *HarCho* were co-expressed separately under constitutively as well as under stress/disease inducible promoter and a synergistic effect of both genes were expected against fungal diseases including FDH.

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4.1.4.2.1 Test of transgenics under Constitutive Promoter:

Three T₁ progenies (*I.A-3*, *I.A-4* and *I.A-6*) were tested for the resistance assays against FHD causal organism and visually effected kernels were couted 21dpi. The middle two spikelets were infected by 200 conidia per spikelet just before fertilization. The infection process was monitored for the next three weeks. There seemed no difference 3dpi but it became clear 9 dpi where on most of the non transgenic and transgenic controls fungi had grown above and below the point of inoculation. Fungi could grow only approximately 2-3 spikelets above the point of inoculation on the tested transgenic lines (with the genes of interest). After 9dpi fungi stoped growing on both controls and tested transgenic lines but on control lines the spikelets above the fungus growing area started fading and this area recovered in lines expressing *HarChit* and *HarCho*. 21 dpi lines (only *I.A-3*, *I.A-4* and *I.A-6* were checked) with the genes of interest showed 41, 14.65 and 11% less visually affected kernels respectively than the controls (The details can be seen in figure 3.23 and 3.24).

4.1.4.2.2 Test of transgenics under Inducible Promoter:

Three T₁ progenies (*I.A-8*, *I.A-11* and *I.A-12*) of the transgenic plants with *HarChit* and *HarCho* under stress/disease inducible promoter were tested with *F. graminearum* and the infection process was followed for the next three weeks. The behaviour of the transgenic plants to *F. graminearum* was same as it was for transgenic plants with *HarChit* and *HarCho* under constitutive promoter. Transgenic progenies showed, 39.40, 16.44 and and 44.64% less visually effected kernels as compared to the control lines (The details can be seen in figures 3.29 and 3.30). The line *I.A-8* although had only *HarCho* but the resistance response of it was at par with the lines containing both the genes of interest.

The resistance criteria are defined as Type-I, Type-II, till Type-V in wheat and barley against FHB of wheat and barley. Type-I resistance is the resistance to the primary infection, Type-II resistance is the resistance offered to the spread of symptoms, Type-III is the resistance offered by the developing kernels, Type-IV is the resistance offered to the toxin production e.g. DON and Type-V is the resistance for the maintenance of total yield (Schroeder and Christensen, 1963 and Mesterhazy, 1995). In the present project point inoculations were done and Type-II and Type-III resisances were recored in the form of reduced spread of fungus in the spikes and the healthier kernels/ visually affected kernals compared to controls in the area above of fungus propagation region (Figure 3.24). The same kinds of results were found by Balconi *et al.* (2007) by expressing b-32 gene (with ribosomal inactivating protein) of maize

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in wheat with antifungal genes. They calculated total amount of b-32 RIP in six independent transgenic lines and found out that there was no relation in the total amount of b-32 protein among six transgenic lines and the decrease of FHB symptoms. They found a difference of upto 30% in disease symptoms compared to control. Many reports have shown the reductions in FHB severity under green house and field conditions by the expression of antifungal proteins. Shin *et al.*, (2008) showed upto 58% reduction to FHB susceptibility under green house conditions but under field conditions only 2 out of 16 lines showed reduced susceptibility. Chen *et al.*, (1999) showed a 63% reduction in FHB susceptibility by over expressing rice thaumatin like protein in wheat. Mackintosh *et al.*, (2007) evaluated the overexpression of three defence response proteins in wheat and observed 34%, upto 36% and upto 49% reduction in disease severity to FHB with α -1-purothionin, tlp-1 and β -1,3-glucanase respectively although in the the field only some of these lines continued with reduced susceptibility. In contrast to these results Anend *et al.* (2003) reported the overexpression of a chitinase gene in wheat that did not result in decrease of disease susceptibility under green house conditions.

It can be seen from the results found in this project and some of the projects completed earlier that it is not possible to get complete resistance against the fungal diseases by overexpressing single or more than one antifungal genes in wheat rather a decrease in the susceptibility is achieved. The same kinds of results have been seen in other crops by introducing defence response/anti-fungal genes. In rice, Kim *et al.*, (2003) co-expressed chitinase and RIP genes and observed a decrease of 30% disease susceptibility against sheath blight. Hendrix and Stewart, (2002) overexpressed chitosanase gene from *Paenbacillus* into tobacco and observed a reduction in disease severity against *R. solani*. Similarly the disease resistance in tobacco and potato plants was improved by the overexpression of the 42 kDa endochitinase from *T. harzianum* (Lorito *et al.*, 1998)

We tried to over express *HarChit* and *HarCho* genes in wheat considering the results of Jash *et al.*, (1995) and Terras *et al.*, (1993) that showed the synergistic effects of the antifungal proteins *in vitro* and *in vivo*. Our results showed a reasonable decrease in disease susceptibility i.e., upto 75% decrease in wheat Powdery Mildew and upto 45% decrease for wheat head scab.

4.2 Role of Glucan Synthase like genes family in wheat:

The family of glucan synthase-like (*GSL*) genes in higher plants has been identified as presumably encoding for callose synthases. The hypothesized function of *GSL* genes is

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supported by homology with the yeast *FKS* genes, which are believed to be involved in callose synthesis. Generally callose is found in papillae and is supposed to play a role in resisting the spread of pathogens in the plants after wounding or pathogen attack. During normal growth conditions, callose is deposited at the cell plate, plasmodesmatal canals, root hair and spiral thickenings in tracheids, sieve plates of phloem elements as well as around pollen mother cells, in pollen grains and in pollen tubes. The family of callose controlling genes has been reported in many plants including wheat and functional analysis of these genes are in progress. This report presents the possible role of three of the members of this family in wheat.

4.2.1 Preparation and Transformation of *Ta-GSL* Knock Down constructs with possible role of the *Ta-GSL* genes in plant development:

The basic principle of RNAi is the production of double stranded RNA that can be recognised by the cell system for degradation. This double stranded RNA can be in the form of viral replicating genome, transposon or any other form of double stranded RNA that can be recognised for degradation. In order to create artificial knock down by genetically transforming the organism with hairpin RNA forming construct, the most important things are the legs of the hairpin. These legs of the hairpin are recognised by the cell system as the double stranded RNA and digested into 22- 24 bp long RNA (siRNA) fragments and these fragments further elicit the system for RNAi machinery to continue. The length of the hairpin should therefore be long enough to initiate the phenomenon. There are different reports about the length of these legs in different organisms ranging from below 100 bp to above 850 bp (Wesley *et al.* 2001). In wheat there are reports on the gene silencing using a fragment of 169 bps, 313 bps and 550 bps (Humanes *et al.* 2006, Folck, 2004 and Regina *et al.* 2005). The knock down efficiency in these reports was seen from 50% to complete knock down. Depending on these results it was decided to make constructs with different lengths of siRNA forming DNA sequences/legs of hairpin. For *Ta-GSL-8* two constructs were made with 122 bps and 230 bps long siRNA forming sequences, for *Ta-GSL-3* the sequence was 152 bps long and for *Ta-GSL-10* a construct containing 230 bps long siRNA forming DNA sequence was used.

All of these constructs were then tried to be transformed into wheat using the protocols described in the section 2.2.4 and only 8 plants were recovered after the transformation experiments by bombarding more than twenty thousand embryos for the entire four construct. Out of these 8 plants only 4 contained the constructs of interest. Two plants with *Ta-GSL-3*

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(152 bp siRNA forming region) construct and two plants for *Ta-GSL-8* (both with 122 bp siRNA forming fragment) construct. These results seem at par with the results obtained for gene silencing experiments of wheat in the beginning by Yan *et al.*, 2004, Loukoianove *et al.*, 2005 and Regina *et al.*, 2005 who got one or two knock down lines but different from Travelia *et al.*, 2006, Folck, 2004 and Humannes *et al.*, 2008 who got sufficient number of knock down lines. These four plants were checked and confirmed for the expression of the RNAi cassette by Northern blot analysis and then advanced to the next generation by sowing the self pollinated seed of the primary transformant. In the T₁ generation plants were checked for the presence and expression of RNAi constructs in all the four lines. After confirming the presence and expression of the constructs in all the four lines the presence of transcript for the relevant genes was evaluated by semiquantitative-RT-PCR using the primers specific to *Ta-GSL-3* and *Ta-GSL-8*. The results showed a reduction in the transcript of the knock down line $\Delta Ta-GSL-3-1$ and $\Delta Ta-GSL-8-1$. The genes did not look completely knock down rather a reduction in the transcription was observed. While the other two lines showed the same level of transcript as was shown by non knock down line Florida (Figure 3.41).

The constructs with 230 bp (For *Ta-GSL-8* and *Ta-GSL-10*) never produced any transgenic plant rather during the tissue culture they gave very little green materials and the calli were never looking healthy. To check whether these constructs are toxic for the plant regeneration a control experiment was done comparing the co-bombardment of RNAi construct and selection marker gene and selection marker gene alone. It was found out that the embryos those were bombarded with selection marker gene and RNAi construct were not looking healthy during tissue culture and they did not produce any transgenic plant while the embryos which were bombarded with the selection marker gene only gave healthy calli and produced embryos which later produced transgenic plants having *BASTA* resistance (figure 3.36). From here, it is concluded that may be these genes have some important role to play in the development of the plant. We may explain the situation by making a hypothesis here; hypothesis is, “May be the genes are completely knock down when we use siRNA forming sequence of 230 bp and this is toxic for plant development so we could not have the development of transgenic plants from these cells or tissues.” At siRNA forming sequence of 122 and 152 bp for *Ta-GSL-8* and *Ta-GSL-3* there was either no reduction in transcript for endogenous genes or there was incomplete knock down although the constructs were always expressing themselves. The plants which we got for RNAi constructs with smaller siRNA forming sequences were normal in their phenotypic growth and development except for the fact that $\Delta Ta-GSL-3-1$ line was late in maturity for 1-2 weeks. The hypothesis looks strong by the fact that the reports

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presented for the same gene family in other plants especially dicot model crop *Arabidopsis* indicates the same results. The functional analysis of *At-GSL-8* and *At-GSL-10* was done with T-DNA disrupted genotypes and RNAi constructs. They were found to have role in male gametogenesis. In the first case it was not possible to get homozygous line for T-DNA insertion and the RNAi lines were highly abnormal with dwarf phenotype. Similarly *At-GSL-5* and *At-GSL-11* were also found to have role in plant development (Jacobs *et al.*, 2003; Töler *et al.*, 2008; Huang *et al.*, 2009).

4.2.2 Role of $\Delta Ta-GSL-3$ and $\Delta Ta-GSL-8$ in FHB resistance:

The entire four knock down lines were tested in the T₁ generation for its susceptibility to *F. graminearum* using the protocols given in the section 2.2.6. Middle two spikelets were inoculated with 200 conidia per spikelet and the infection process was monitored for the next three weeks. 3dpi there was no visible infection in the knock downs and control line Florida plants but after that fungus started growing rapidly and the difference between knock downs and control line Florida started growing. Fungus almost stopped growing after 9dpi both in control and knock down lines. Going past 9 dpi the spikelets above and below the area of fungal growth kept fading away at different rates. Total number of visually effected kernels were counted in between 18-21dpi. The results found showed the continuation of the molecular analysis of the knock down lines. The line $\Delta Ta-GSL-3-1$ showed the maximum susceptibility to *F. graminearum* compared to the control line Florida. Control line showed on an average 42.05% visually affected kernels while the knock down lines ($\Delta Ta-GSL3-1$, $\Delta TaGSL3-3$, $\Delta TaGSL8-1$ and $\Delta TaGSL8-3$) showed 60.14%, 46.25%, 49.79% and 46.431% of visually effected kernels respectively. For every 100 affected kernels $\Delta Ta-GSL-3-1$ showed 144 visually affected kernels and $\Delta Ta-GSL-8-1$ showed 118. These were the two lines which also showed a significant reduction in the level of transcript for the endogenous *Ta-GSL3* and *Ta-GSL8* genes when the gene specific primer were used in the semi quantitative RT-PCR. The other two lines ($\Delta TaGSL3-3$ and $\Delta TaGSL8-3$) who showed the expression of RNAi constructs but not the reduction in the transcript of endogenous *Ta-GSL3* and *Ta-GSL8* genes showed very less increase in the susceptibility (110 visually affected kernels in both lines for every 100 visually affected kernels of the control) against FHB. It can be seen in the figure 3.41 that maximum reduction in *Ta-GSL3* knock down is shown by $\Delta TaGSL3-3$. Maximum increase in FHD susceptibility is also shown by the same line. Same co-incidence is also seen for other three lines. Depending upon these results we can blame the increased susceptibility to reduction in the *Ta-GSL3* and *Ta-GSL8* gene transcript. We speculate that atleast these two

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members of *GSL* family of genes in wheat have some role to play in resistance against fungal pathogen *F. graminearum*. In terms of plant defence in arabidopsis *At-GSL5*, *At-GSL-6* and *At-GSL-11* have shown their involvement. It is reported that the transcript levels of these three arabidopsis genes increases in the leaves upon inoculation of *Blumeria graminis* spores. The functional analysis of *At-GSL5* by gene knock down showed that the Glucan Synthase encoded by this gene is required in the papillary callose formation and also in the salicylic acid formation pathway. The plant growth was slightly stunted in general in the knock downs. The growth of the several species of powdery mildews and *Perrenospora parasitica* was ceased in *At-GSL5* knock downs. This is some thing reverse to our results. The results found with *At-GSL5* reject the commonly found model of the involvement of callose in disease resistance (Jacobs *et al.*, 2003, Nishimura *et al.*, 2003, Enns *et al.*, 2005 and Dong *et al.*, 2005) while our results support the model.

There are a lot of things to be found about the *GSL* gene family of plants. Results of functional analysis of the some members of this family in *Arabidopsis* indicate their involvement in cell division, male gametogenesis and plant development and disease resistance. In wheat, according to our knowledge this is the first report about functional analysis of any members of *GSL* gene family. Our results indicate the involvement of this family both towards disease resistance and the plant development. Future studies with other genes of the family and further details of the presently discussed genes will uncover what this family offers for wheat in general.

4.3 Candidate genes for Disease Inducible Promoter:

The success of a transformation experiments depend upon the expression of the gene/genes with out affecting the innate machinery of the transformed plants. In the past constitutive promoters have been used to express transgenes in different plants. In some recent experiments it is observed that the use of constitutive promoter some times negatively interacts with plant systems resulting in less number of regenerants as well as abnormal plants (Cao *et al.*, 1998; Piererse and Loon, 2004). The probable reason of such abnormalities can be the extensive reprogramming in the transgenic plants upon expression of a foreign protein, especially in the parts of plant where this is absolutely not needed (Gurr and Rushton, 2005). The sole solution of this problem is the identification of inducible or tissue specific promoters. In the wake of the recovery of the less number of knock down plants in this project it was decided to find out the disease inducible genes in wheat which can be used later for the identification of disease inducible promoter. For the identification of the differentially

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expressed genes upon disease infection some studies have reported the micro array analysis using c-DNA from the control and infected plants at different time points. These experiments revealed the up-regulation of various biotic and abiotic stress related genes in wheat (Pritsch *et al.*, 2000 , Kruger *et al.*, 2003, Kong *et al.*, 2003 and Boddu *et al.*, 2005).

In the present experiment 10 contigs were selected from barley gene chip reported by Eichmann *et al.*, 2006 and provided by Prof. Dr. W. Schaeffer (University of Hamburg, Germany). These ten sequences were used to find out the homologous genes/sequences in wheat by BLAST search in the genomic data base. Additionally two ESTs were selected from Kruger *et al.*, 2002 which are highly expressed upon *F. graminearum* infection at early time points in semi-resistant wheat genotype *Sumai-3*. The homologous sequences found in wheat mainly were reported as pathogenesis and defence related genes. All the twelve genes were tested at first with rt-PCR using non infected control; mock inoculated control and *F. graminearum* inoculated wheat spikes (susceptible genotype Florida) at 12 hai, 24 hai, 48 hai, 72 hai, 96 hai, 120 hai and 144 hai. The results showed the over expression of all the 10 wheat genes on *F.graminearum* inoculation but there was no reduction or increase in both of the genes reported from *Sumai-3*. May be these two genes which are up-rregulated at early time points are the part of “*Sumai-3*” semi resistance mechanism against FHB compared to “Florida”.

Out of the 10 genes 4 genes were selected for further analysis with Northern Blot analysis. The rest of the 6 genes were discarded based on relatively low expression levels or the expression on mock inoculation along with up-regulation on *F. graminearum* infection. The results of the Northern Blot analysis showed that all the four genes are up-regulated upon *F. graminearum* infection and the maximum level of the transcript is achieved at 72 hai and 96 hai which keep on decreasing till 144hai. Genes/sequences “*pk0023*” and “*oxalate oxidase precursor gene*” showed no expression on the mock control and till 24 hai and 48 hai respectively of *F. graminearum* while “*Ta-Chitinase*” and “*PDR like ABC transporter gene*” showed some back ground activity on mock control. “*pk0023*” and “*Ta-Chitinase*” showed high expression as compared to the “*oxalate oxidase precursor gene*” and “*PDR like ABC transporter gene*”. Depending upon these results it can be said that “*pk0023*” and “*Ta-Chitinase*” are the best candidates for disease inducible promoter although some background activity is present at “*Ta-Chitinase*”. When absolutely no expression is needed under normal conditions with high expression under disease then “*pk0023*” is recommended. “*Oxalate oxidase precursor gene*” is although completely inducible but the expression level is too low under disease as well.

CONCLUSION AND OUTLOOK

CONCLUSION AND OUTLOOK

The goal of this study was to analyse the role of some endogenous and exogenous genes in wheat disease resistance so that a future strategy can be devised for the development of a genotype that can be resistant against more than one disease pathogens. For this purpose a couple of antifungal genes (*HarChit* and *HarCho*) from *Trichoderma harzianum* were co-transformed into wheat genotype “Florida” under the control of constitutive as well as inducible promoter. 4 lines showed the co-expression of both the genes under constitutive promoter while five lines showed co-expression under disease/stress inducible promoter. While 2 lines and one line showed single gene integration under constitutive and inducible promoter respectively. The infection assays performed with *E. graminis* f.sp. *tritici* and *F. graminearum* showed a reduction in disease development and spread of upto 75% compared to the non transgenic control. From here it can be concluded that these two anti-fungal genes do have a role in the resistance against fungal diseases in wheat and the transgenic lines developed in this project can be used in the breeding programmes (after pathological testing in the field) for the development of disease resistant cultivars.

As a second strategy three members of the Glucan Synthase Like (*GSL*) family of genes were tried to knock down for the function analysis with respect to fungal diseases. RNAi constructs were made for all the three genes at different sizes of the siRNA forming fragment of the constructs. Transgenic plants containing RNAi constructs with only short siRNA forming fragments could be recovered. Two out of four transgenic lines showed partial knock down of *Ta-GSL3* and *Ta-GSL8*. Pathological analysis of all the four lines showed that only two lines with partial knock of genes showed an increase in the susceptibility to *F. graminearum* compared to non transgenic. From these results it can be concluded that perhaps short siRNA forming DNA fragments can only partially knock down a gene in wheat while larger siRNA forming DNA fragments in the range of 230 bp can completely knock out a gene. The complete knock down is toxic for wheat plant development. The increments in the susceptibility of the partial knock downs indicate that these genes are not only have a role in plant development but also the play a role in the resistance against fungal diseases. In future experiments it will be interesting to see if the over expression of these genes can enhance resistance against fungal diseases.

The non/less recovery of transgenic plants with RNAi cassettes under constitutive promoter gave rise to the decision of finding out some genes which can be used in the identification of disease inducible promoter in future. We found out 4 disease inducible genes whose promoter can be sequenced and used in the future experiments of such nature.

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ZUSAMMENFASSUNG

Weizen (*Triticum aestivum* L.) stellt eines der wichtigsten Getreide der Welt dar. *T. aestivum* weist die größte Anbaufläche weltweit auf und ist nach Mais am zweitwichtigsten in der globalen Getreideproduktion. Weizen ist anfällig für viele Krankheiten, die meistens durch Pilze ausgelöst werden. In kalten und feuchten Klimazonen sind zum Beispiel Ährenbleiche Fusarium Head Blight (FHB) und echter Weizenmehltau Auslöser ertragsmindernder Krankheiten. Mit Hilfe von klassischen Züchtungsprogrammen konnten Sorten entwickelt werden, welche begrenzt Resistenzen gegen FHB oder echtem Weizenmehltau aufwiesen. Aufgrund der mutierenden Pathogene und der vertikalen Natur der Resistenz sind diese Sorten nach einigen Jahren wieder anfällig für diese Pathogene. Die Fortschritte in der Gentechnik eröffnen den Wissenschaftlern und Pflanzenzüchtern neue Wege, um Genotypen zu entwickeln, welche eine stabile Resistenz gegen mehrere Pathogene ohne Artspezifität aufweisen. Um dies zu erreichen wurden in dieser Arbeit zwei unterschiedliche Ansätze verfolgt. Zum einen wurden zwei antifungale Gene (*HarChit* und *HarCho*) unter der Kontrolle eines konstitutiven *Ubi*-Promotors und eines Stress-induzierbaren *Vst*-Promotors koexprimiert. Zum anderen wurden drei Mitglieder (GSL3, GSL8 und GSL-10) der Glucan-Synthase-Ähnlichen-Genfamilie ausgeschaltet, um deren Funktion in der Krankheitsresistenz zu untersuchen.

Insgesamt konnten neun transgene Linien regeneriert werden, welche eine Kointegration und Expression der Gene *HarCHit* und *HarCho* aufwiesen. In vier Linien wurden diese Gene durch einen konstitutiven *Ubi*-Promotor kontrolliert und in fünf Linien wurden diese Gene durch den induzierbaren Promotor *Vst* kontrolliert. (Drei transgene Regenerate enthielten nur *HarChit* oder *HarCho*.) Das Integrationsmuster wies sowohl Einzel- als auch Mehrfachintegrationen der beiden Gene auf. Die Anzahl an Kopien von *HarChit* (1 bis 3) und *HarCho* (2 bis 10) variierten. Die Verwendung eines induzierbaren Promotors schien keinen Einfluss auf die Transformation zu haben. Über Infektionsanalysen konnte eine Abnahme der Anfälligkeit gegenüber der untersuchten Pathogene beobachtet werden. Die Anfälligkeit gegenüber *E. graminis* sank um 75 %, wohingegen die Anfälligkeit gegenüber *F. graminearum* um 58 % sank. Alle Primärtransformanten, mit wenigen Ausnahmen, wiesen ein normales Wachstum auf.

Bei nur vier transgenen Linien (keine für *Ta-GSL-10*) konnte eine Geninhibierung der *GSL*-Gene unter Verwendung von etwa 150 bp großen siRNAs für die DNA-Fragmente in den RNAi-Konstrukten beobachtet werden. Wurden größere siRNAs für die DNA-Fragmente in

den RNAi-Konstrukte verwendet, konnten keine Pflanzen regeneriert werden. Nur zwei von vier transgenen Regeneraten (1 für *GSL3*, 1 für *GSL8*) zeigten eine Reduktion der Genexpression in den T₀- und T₁-Generationen. Infektionsanalysen mit *F. graminearum* zeigten eine Zunahme der Krankheitsanfälligkeit bis zu 60 % bei *Ta-GSL3*-Inhibierung und bis zu 40 % bei *Ta-GSL8*-Inhibierung. Dadurch konnte der Zusammenhang zwischen diesen Genen und der Krankheitsresistenz nachgewiesen werden. Aufgrund der Schwierigkeiten der Geninhibierung unter Verwendung von RNAi-Kassetten unter der Kontrolle von konstitutiven Promotoren, wurde nach Genen gesucht, die durch Krankheiten induziert werden. Es konnten vier Gene identifiziert werden, welche durch Infektionen mit *F. graminearum* induziert wurden. Die Promotoren dieser Gene könnten in zukünftigen Versuchen verwendet werden.



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APPENDICES

Appendix-1 Preparations of Gold Suspension and DNA Coating

(a) Gold Suspension

- (i) Wash 40mg gold in 1ml absolute ethanol 3 times by vortexing
- (ii) Wash gold in sterile ddH₂O 3 time by vortexing
- (iii) Re-suspend gold in 1ml sterile ddH₂O
- (iv) Ultrasonicate for 20 sec.
- (v) Freeze in 50 μ l aliquots (approx. 2mg/50 μ l) in 2ml Eppendorf tubes.
- (vi) Sonicate before use

(b) DNA Coating

- (i) Add 5-6 μ l of μ g/ μ l plamid DNA into the 50 μ l gold suspension and vortex.
- (ii) In Eppindorf lid, add 50 μ l 2.5M CaCl₂ and 20 μ l 0.1M spermidin.
- (iii) Close lid and vortex immediately and set on ice for 5sec and centrifuge for 30sec.
- (iv) Pipette-off the supernatant and re-suspend in 250 μ l absolute ethanol.
- (v) Centrifuge for 5sec and pipette-off the supernatant.
- (vi) Re-suspend DNA-coated gold in 240 μ l absolute ethanol.
- (vii) Load 3.5-10 μ l on macrocarrier for particle bombardment.

Appendix-2 GUS Staining Buffer and Histochemical staining

A. Preparation of GUS staining solution:

- (i) 80 mM sodium phosphate buffer [pH 7.0]
- (ii) 0.4 mM potassium ferricyanide.
- (iii) 0.4 mM potassium ferrocyanide
- (iv) 8 mM EDTA
- (v) 0.05% Triton X-100
- (vi) 0.8 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-glucuronide
- (vii) Filter-sterilize using 0.2 micro filter.
- (viii) For 100 ml of GUS staining solution, add 25 ml MeOH (to give 20% MeOH concentration)
- (ix) Before first use, add 60 ul of Triton-X for each 100 ml.

B. Histochemical assay:

- 1 Dip the sample in GUS staining solution.
- 2 Incubate for one hour to overnight at 37C.
- 3 Remove GUS staining solution.
- 4 Add 70 – 100% ethanol.
- 5 Incubate at room temperature to remove chlorophyll.
- 6 Repeat steps 4 and 5 several times

Appendix-3 T Tissue Culture Media for transformation

	Callus induction medium (CIM)	Selection medium (SM-II)	Regeneration Medium SM-III	Rooting medium ¹ / ₂ MS
Macroelements (mg/l)				
NH ₄ NO ₃	1650	1650	1650	825
KNO ₃	1900	1900	1900	950
KH ₂ PO ₄	170	170	170	85
CaCl ₂ .2H ₂ O	440	440	440	220
MgSO ₄	370	370	370	185
Na-EDTA	373	373	373	186.5
FeSO ₄ .7H ₂ O	278	278	278	139
Microelements (mg/l)				
H ₃ BO ₃	6.20	6.20	6.20	3.1
MnSO ₄ . 4H ₂ O	11.20	11.20	11.20	5.6
ZnSO ₄ . 4H ₂ O	5.6	5.6	5.6	2.8
KI	0.83	0.83	0.83	42.5
NaMoO ₄ . 2H ₂ O	0.25	0.25	0.25	0.125
CuSO ₄ . 5H ₂ O	0.025	0.025	0.025	0.0125
CoCl ₂ . 6H ₂ O	0.025	0.025	0.025	0.0125
Sucrose (g/l)	30	30	30	15
2,4-D (mg/l)	2mg/l	2mg/l	0.1mg/l	
BASTA	-	3mg/l	3mg/l	-
Gelrite	0.3%	0.3%	0.3%	0.3%

Osmotic medium consists of basic M.S. Macro salts, Micro salts and and sucrose @ 479.22g per litre.

Appendix-4 DNA extraction Buffer:

End Concentration	Stock solution	Quantity for 200ml
1% Lauryl Sarkosyl	30	6.6ml
100mM Tris-HCl	1M	20ml
100mM NaCl	1M	20ml
10mM EDTA	0.5M	4ml

Appendix-5 peqGold Trifast RNA Isolation

1	Homogenisation	1.0 ml peqGOLD TriFast™ + 50-100 mg leaf sample
2	Phase separation	Homogenate + 0.2 ml Chloroform
3	RNA-Precipitation	Watery Phase + 0.5 ml Isopropanol
4	RNA washing	1 ml 75 % Ethanol
5	Dissolving RNA	Formamide, 0.5 % SDS or Water

Appendix-6 Protocol for First-strand cDNA Synthesis (PCR template generation with M-MuLV Reverse Transcriptase/ RT-PCR)

1. The following mixture was prepared in a reaction tube and kept on ice.

Template	Quantity for PCR
Total RNA	1.0-5µg

Primer	Quantity for PCR
Oligo(dt)18	0.5 µg

Water (Nuclease free)	To 11.5 µL
-----------------------	------------

2. Incubated the mix at 70°C for 5 minutes and chilled on ice

3. Added the following in the order indicated

5X reaction buffer for Reverse Transcriptase	4.0 µL
dNTP Mix, 10 mM each	2.0µL (1.0 mM final concentration)
RiboLock™ Ribonuclease Inhibitor	0.5 µL (20 u)
DEPC-treated Water	to 18 µl

4. Incubated the mixture at 37°C for 5 minutes.

5. Added 40 units of reverse transcriptase, incubated the reaction mixture, containing oligo(dT)₁₈ **37°C** for 60 minutes.

6. Stopped the reaction by heating at 70°C for 10 minutes and was chilled on ice.

Synthesized cDNA was always used directly for PCR reactions.

Appendix-7. PCR Reagents.

Reagent	Final Concentration
Sterile deionized water	to reaction volume
10X <i>Taq</i> buffer	1X
2mM dNTP mix	0.2mM of each
Primer I	0.1-1 μ M
Primer II	0.1-1 μ M
<i>Taq</i> DNA Polymerase	1.25U/50 μ l
25mM MgCl ₂	1-4mM
Template DNA	10pg-1 μ g

Appendix-8 SOUTHERN BLOTTING

1. 25 μ g of genomic (hexaploid genome) DNA per sample was loaded in 0.8% agarose gel. Et Br was also added in the gel to give color to the bands under UV.
2. Incubated the gel in 0.25% HCL for 5 minutes to break the bigger DNA fragments into smaller ones in order to ease out the transfer of DNA to Nylon membranes later on.
3. Incubated in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 minutes
4. Incubated in neutralization buffer (1.5 M NaCl, 0.5 M Tris HCl, 0.001M ADTA, pH 7.2) for 2X 15 minutes.
5. For blotting Hybond N membrane was always used whose background is light than the positive membrane whose detection takes normally longer time.
6. After removing the blots the membranes were always swirled in 2X SSC for 10 seconds to wash off the adhered agarose. Subsequently the membrane with DNA side toward upwards was dried in 3 MM Whatman paper for 15 minutes. The fixing of DNA to membrane was done by UV cross linking in strata linker on 1200 KJ.
7. Prehybridization is done in bags or bottles. We use hybridization buffer indicated on page 5 with formide and 50 μ g/ μ L Herrings sperm DNA. Pre-hybridization is done for 4-6 hours. Over night hybridization is done in the same buffer adding 5ng probe /ml of hybridization buffer (estimate probe concentration by PCR marking (Page 7) over the Gel. The hybridization solutions plus probes can be used for 20 hybridization reactions. After approximately 4 h exposure higher background should be available, we should dilute hybridization solutions to some extent. For a 200 cm² membrane I use 30 ml pre hybridization solution and approximately 10 ml hybridization solution. Pre and hybridization is hybridized at 42 °C. We usually use PCR probes; these are clearly more sensitive than random primed labelling. For example protocols for bar and Gus genes are settled (page 7)
8. Wash membrane in solutions W1-W3 (page 5) by 65 °C as written on page 6.
9. Make B2 buffer (page 5) by 65 °C, shake for approximately 30 minutes and pour through a folded filter paper. For a 200 cm² membrane we use 200 ml B2.

10. Mix 1:7500 antibodies in 80 ml (for 200 cm²) B2 and push the entire solution through 1.2 µm filter to destroy all the antibody aggregates.
11. Wash 3X 15 minutes or longer in WB.
12. Place in B2 for 5 minute.
13. Place membrane in a cut and open plastic bag and shift with 5 ml B2 plus CSPD (1:100) for 5 minutes.
14. Take membrane from bag and place on a 3MM whatman paper, with a second 3MM paper shortly dry. Apply only shortly upward and subsequently wrap the membrane. Drying must be done very fast. Membrane should not be so damp and not so dried. (blurred signals means low sensitivity)
15. Incubate wrapped membrane for 15 minutes on 37 °C subsequently printing Film. We make first exposure normally for 4h. With no or very thin back ground can also be exposed over night.

Solutions used for DIG detection of Southern.

Pre-hybridization and Hybridization buffers were used from company Roche available under catalog # 1 603 558 (01.11.2006)

DIG-11-dUTP (alkali-labil) 125 nmol from company Roche, catalog # 11 573 179 910 (01.11.2006)

Washing Solutions.

Solution	concentration	Stock used for 1L
W1	2x SSC	100mL 20x SSC
	0.1%SDS	10mL 10%SDS
W2	0.5x SSC	25mL 20x SSC
	0.1% SDS	10mL 10% SDS
W2	0.2x SSC	10mL 20x SSC
	0.1% SDS	10mL 10% SDS

Washing Buffer (WB) B1-Buffer + Tween 20 (0.3%)

10x B1- Buffer:	1000M Maleic Acid	116.01g/L
	150mM NaCl	81.66 g

Adjust pH with NaOH to 7.50. On dilution of the buffer to 1x, pH has to be confirmed again.

B2 Buffer 800 mL B1- Buffer 1x + 8g Blocking Reagent
 Blocking Reagent 50 g from Company Roche # 12562300
 Heat it at 65°C and then filter.

B2 with antibody: Add 21.5 µL Antibody solution in 200 B2 Buffer. (Eppi. containing anti body solution should be centrifuged 2-3 minutes before use)

Anti-Digoxigenin-AP, FAB fragments

Company Roche # 11 093 274 910 150U (200µL)

B3-Buffer (always fresh prepared)	Stock Used for 50 mL
100 mM Tris-HCl (pH 9.5)	5 mL 1M Tris -HCl pH 9.5
100 mM NaCl	1 mL 5 M NaCl
50mM MgCl ₂	2.5 mL 1 M MgCl ₂

B3 + CSPD-Substrate: dilute CSPD- Substrate 1:200 in B3 and put it on ice

(75µL CSPD for 15 mL B3)

CSPD from company Roche # 11 759 043001

Appendix-9 Recipe for 1 liter SNA media:

Chemicals	Amount	Final concentration
KH₂PO₄	1g	0.1%
KNO₃	1g	0.1%
MGSO₄ X 7H₂O	0.5g	0.05%
KCL	0.5g	0.05
GLUCOSE	0.2g	0.02%
SUCCHAROSE	0.2g	0.02%
H₂O	1L	99% approx.
GRANULATED AGER	16g	1.6%

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CURRICULUM VITAE

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Research project: “Reverse and forward genetic approaches for the development of disease resistant wheat (*Triticum aestivum*L.)”. The focus of the study was to use Molecular Biology tools for the development of disease resistant wheat.

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