TRANSFORMATION OF TOBACCO (*Nicotiana tabaccum)* WITH ANTIMICROBIAL *pflp* GENE AND ANALYSIS OF TRANSGENIC PLANTS

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ABSTRACT

TRANSFORMATION OF TOBACCO (*Nicotiana tabaccum)*
WITH ANTIMICROBIAL *pflp* GENE
AND ANALYSIS OF TRANSGENIC PLANTS

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The objective of this study was to transform sweet pepper ferredoxin-like protein (PFLP) gene, which has antimicrobial properties, to tobacco and investigate the disease resistance abilities of transgenic tobacco. This protein interacts with another protein, harpin that is produced by the bacteria which is invading the plant tissues, and stimulates hypersensitivity response in plants, thus the spreading of disease is limited.

Gene transfer was achieved to tobacco by *Agrobacterium*- mediated method and with indirect organogenesis; the explants were grown on selective media and

then transferred to jars and pots respectively. Molecular and genetic analyses such as PCR, RT-PCR, Sequence Analysis and Northern Blot, were performed with plants which their seeds survived and grew on selective medium and also gave positive reactions for GUS histochemical assay.

Finally, with putative transgenic plants, some hypersensitive response assays were carried out with *Pseudomonas syringae* and it was observed that the recovered plants showed hypersensitive response (HR) in the preliminary tests. These results indicated that putative transgenic tobacco plants which carry *pflp* transgene, can be used in disease resistance studies.

Keywords: ferredoxin-like protein, hypersensitive response (HR), Transgenic, *Agrobacterium tumefaciens*

ÖZ

ANTİMİKROBİYAL *pflp* GENİNİN TÜTÜNE AKTARILMASI VE TRANSGENİK BİTKİLERİN GELİŞTİRİLMESİ

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Bu çalışmanın amacı antimikrobiyal özelliği olan tatlı biber ferredoksin-benzeri protein (PFLP) geninin tütüne aktarılması ve transgenik tütünlerin çeşitli bakteriyel hastalıklara dirençlerinin incelenmesidir. Bu protein, bakterilerin bitkiyi istila sırasında ürettiği harpin adlı proteinle etkileşerek bitkide hiperduyarlılık tepkisi başlatmaktadır, böylece hastalığın yayılması engellenmektedir.

Agrobacterium yöntemiyle tütünlere gen transferi yapılmış olup indirekt organogenesisle tütünler seçici ortamda büyütüldükten sonra sırasıyla kavanozlara ve saksılara konulmuşlardır. Tohumları higromisinli ortamda büyüyen ve GUS

boyamasıyla olumlu renk veren bitkiler ile PCR, RT-PCR, Sekans Analizi ve Northern Blot gibi moleküler ve genetik analizler yapılmıştır.

Çalışmanın bu kısmından sonra transgenik adayı bitkiler üzerinde çeşitli patoloji deneyleri yapılmıştır. Öncül deneylerde, *Pseudomonas syringae* bakterisine karşı yapraklarda hiperduyarlılık tepkisi gözlenmiştir. Bu sonuçlar göstermektedir ki *pflp* genini taşıyan tütün bitkileri hastalık direnci çalışmalarında kulanılabilirler.

Anahtar kelimeler: ferredoksin-benzeri protein, hiperduyarlılık tepkisi, Transgenik, *Agrobacterium tumefaciens*

To the spirit of my ancestors

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LIST OF ABBREVIATIONS

Avr	Gene coding for Avirulence Proteins in pathogens
BA	Benzylaminopurine
Bt	*Bacillus thrungiensis*
Cf	Cefotaxime
Cry	Gene coding for Crystal Protein in *Bacillus thrungiensis*
cv	Cultivated Variety
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediamine Tetra Acetic Acid
GM	Genetically Modified
GMO	Genetically Modified Organisms
GUS	β-D-glucuronidase
H	Hygromycin
HR	Hypersensitive Reaction
IBA	Indole Butyric Acid
JA	Jasmonic acid
K	Kanamycin
MS	Murashige and Skoog Basal Medium
OD	Optical Density
PCR	Polymerase Chain Reaction
pflp	Gene coding for Plant Ferrodoxin-like Protein
PFLP	Plant Ferrodoxin-like Protein
PR	Pathogenesis-Related
PRSV	Papaya ring spot virus
R	Genes coding for Resistance Proteins in plants
ROS	reactive oxygen species
rpm	Revolution per Minute
SA	Salicylic acid
SAR	Systemic Acquired Resistance

SEM	Standard Error of Mean
TAE	Tris-Acetate-EDTA
T-DNA	Transferred DNA
TMV	Tobacco mosaic virus
uidA (gusA)	Gene coding for β-D-glucuronidase
vir	Virulence genes
X-GlcA	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic Acid
XR	Rapid flux of ions across the plasma membrane
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

1.1. Transgenic Plants and their Application in Agriculture

1.1.1. Improvement of Crops and Genetic Modifications

For centuries, genetic manipulation of plants has been carried out by applying conventional plant breeding methods. Most of these studies were not involved with a single gene; rather they have dealt with a large chromosome piece which contains the desired gene. As a result, the transferred chromosome contained many other genes which, generally, might not be desired (Murray, 1993).

Plant breeding principles usually consist of identifying and selecting desirable traits and combining these into one individual plant. For generations, the superior traits are selected as well as the undesired traits are discarded. By this way, breeders have been able to create new varieties that have desirable traits, such as color, shape and flavor. But plant breeding needs very long time to obtain a satisfactory product. Today, there exists a branch of biology which consists of multiple methodologies for developing superior plants: plant biotechnology.

A sub-branch of plant biotechnology, Transgenic Plant Technology, aims to transfer genes from one species to another either related or not. The process of introducing a gene into an organism via recombinant DNA technology is known

as transformation and recovered plant species are called as transgenic plants or genetically modified (GM) plants.

Gene transformation studies for plants have started during the early 1980s (Lamb and Beachy, 1989). During that time, the main aim was to analyze the structure and function of genes. But after a while, the purpose of these studies became; improved product yield and quality, increased resistance to diseases, increased resistance to biotic stress factors such as nematodes, insects and fungus, and increased resistance to various abiotic stresses factors such as drought, high contents of salt and heavy metals in soils.

1.1.2. Gene Transfer Techniques for Plants

Today, there exist many plant transformation techniques which can be classified into two groups as direct and indirect gene transfer Direct transformation techniques consist of microprojectile bombardment, protoplast fusion, electroporation, and microinjection where as indirect transformation techniques includes viral vector mediated and *Agrobacterium*-mediated gene transfer techniques. Frequently used techniques are *Agrobacterium* mediated delivery and microprojectile bombardment.

The indirect gene transfer techniques includes the plant viruses and *Agrobacterium*-mediated gene transfer. Transformation with the former one, plant viruses, is much easier than *Agrobacterium-mediated* transformation, much simpler than protoplast transformation, and much cheaper than particle bombardment since they naturally infect intact plant cells and they multiply to high copy numbers in the plant cell which results in high expression. But plant viruses do not integrate into the genome and therefore have little value as integrative transformation vectors. They also do not pass through the gametes, so their effects are not heritable through meiosis (Twyman *et al.*, 2002)

Nowadays, the soil bacterium *Agrobacterium tumefaciens* is the most commonly used tool in gene transformation studies for majority of dicotyledonous plants. It is based on the transfer of foreign DNA by using natural gene transfer capacity of *Agrobacterium* species (Horsch *et al*., 1985). *Agrobacteria* are gram-negative rod-shaped bacteria that are abundant in soil and classified in genus *Rhizobium* and in family *Rhizobiaceae*. With these bacteria, transgenic plants have been obtained nearly for all cultured species. The satisfactory results for monocotyledonous species could not been achieved at the beginning however with the development of binary vectors, gene transformation by *Agrobacterium* gave efficient results for maize **(**Ishida *et al.,* 1996) , rice (Hiei *et al.,* 1997), barley (Tingay *et al.,* 1997) and wheat (Cheng *et al.*, 1997).

Direct gene transfer techniques do not rely on biological vectors like plasmids and viruses, and naked DNA is directly used. One example to direct gene transfer is protoplast fusion. Protoplasts are plant cells from which the cell walls have been removed enzymaticaly or chemically which make it easier to transfer foreign DNA into cells either by treatment with particular chemicals, or by the use of high voltage electric shocks. The chemicals polyethylene glycol (PEG) and polyvinyl alcohol, together with divalent cations such as Ca^{+2} and high pH, induce protoplasts to take up naked DNA (Paszkowski *et al.*, 1984).

Electroporation is another direct gene transfer technique, which has been developed for mammalian cell transformation, and now adapted to both monocot and dicot species. The application of strong electrical field and high voltage electrical pulses to solution, containing cells and DNA creates reversible pores in the cell membrane that allows the entry of the genes (Fromm *et al.,* 1985).

Microinjection which is commonly used for animal cells is another method and based on the transfer of naked DNA molecules to intact plants or protoplasts by mechanical ways (Crossway *et al.,* 1986). It employs the use of fine micro-capillaries to transfer a specified number of genes into cytoplasm or directly into

nucleus of cells. This method is more suitable for stable transformation rather than transient gene expression but it is labor intensive, has a high cost and number of transformants produced at the end are relatively low compared to other methods of gene delivery.

Microprojectile bombardment, which is also known as biolistics or gene gun method, is simple both conceptually and in practice. Typically, plasmid DNA is prepared by standard methods and precipitated onto tungsten or gold particles using $CaCl_2$. Spermidine and PEG are included to protect the DNA during precipitation, and the particles are washed and suspended in ethanol before drying onto Mylar aluminized foil. This is fired against a retaining screen that allows the microprojectiles through, to strike the target tissue. Once in the cell, the genetic material is transported to the nucleus where it is incorporated into the host genome (Klein *et al.,* 1987). Particle bombardment is widely used because it circumvents major limitations of the *Agrobacterium* system such as the species limitation.

1.1.3. Agricultural Practices of GM crops

Today, many millions of hectares of commercially produced transgenic crops such as, soybean, maize, cotton and canola has been grown annually in a number of countries, including the United States of America (49,8 million hectares), Argentina (17,1 million hectares) , Brazil (9,4 million hectares) and Canada (5,8 million hectares) (James, 2005). Total global area of crops reached 90 million hectares in 2005.

Currently, one of the most commonly used application area of transgenic plants is developing resistance to weeds, which compete with crops and decrease the yield. About two decades ago, the first transgenic plant that is resistant to the herbicide Basta was achieved (De Block *et al*., 1987). In 2004, more than 60% of

produced soybean around the world is transgenic which are known as Roundup Ready and Liberty Link varieties (James, 2004).

Another area is generation of transgenic plants that are resistant to insects (Dulmage *et al*., 1981). *Bacillus thrungiensis* (Bt) forms spores carrying crystal proteins that are toxic to various group insects. Insect resistance was achieved in transgenic plants by transforming *Cry* genes of *Bacillus thrungiensis* and expressing high-levels of this toxic protein (Mazier *et al.,* 1997). In 1995, the first insect resistant transgenic crops; corn, cotton and potato; expressing Cry proteins were approved for market release in the US. Also, trypsin proteinase inhibitors (Alfonso-Rubi *et al.,* 2003) and biotin-binding proteins (avidin and streptavidin) (Markwick *et al., 2003)* are used to generate insect resistant transgenic plants. Besides insects, conferring resistance against nematodes, that cause an estimated 10% to 25% annual reduction in crop yield, is another agricultural application. *R*-genes targeted against nematodes from potato have been shown to confer nematode resistance when susceptible varieties have been transformed with these genes (Paal *et al.*, 2004).

Abiotic stresses such as; drought, salt, heat, cold, chilling and heavy metals also affect crop productivity. Genetic modifications have been carried out and transgenic plants with enhanced tolerance to environmental stress have been developed even at gene regulation level. Constitutive expression of the stress-response transcriptional coactivator multiprotein bridging factor 1c (MBF1c) in *Arabidopsis thaliana* enhances the tolerance of transgenic plants to heat and osmotic stress (Suzuki *et al., 2005)*

Improving the nutritional quality of plants is another purpose of generating GMOs. Golden Rice is the most popular product of this strategy. With the introduction of new genes to rice, transgenic rice exhibits an increased production of beta-carotene so may help to overcome vitamin A deficiency in children (Ye *et al*., 2000).

1.1.4. Disease Resistance and Transgenic Plants

Attacks by plant pathogens always have been a threat to agricultural production. Plant diseases have had historical implications, where one of the most well known examples is the potato famine in Ireland of 1845-1847 caused by *Phytophthora infestans*, which spurred a huge wave of emigration to North America. In 1880s, the coffee industry in Ceylon was completely destroyed due to *Hemileia vastatrix* and a century later the same fungus hit many countries in South and Central America.

Agriculture worldwide suffers from production losses due to pathogens and pests. The crop losses in 1988-1990 of eight principal food and cash crops, which are grown on about 50% of the global cultivated land, was estimated to 29% of the attainable production, corresponding to 167 billion US$. It is estimated that without the application of various crop protection measures, crop losses due to pathogens and pests globally would have reached 40% of the attainable production (Oerke *et al.*, 1999).

Agrochemicals are commonly used to control many plant pests and diseases. In 1990, about 19 billion US$ were spent globally on insecticides and fungicides (Oerke *et al.*, 1999). Other alternatives, or complements, to protect plants include phytosanitary measures such as healthy seed, appropriate crop rotation and tillage methods.

Another very important factor in controlling plant pathogens is the use of resistant cultivars. Classical plant breeding is continuously aiming at improving plant resistance to diseases and pest attacks by introgression of resistance from the same or closely related species. However, in many instances there are no sources of resistance available or the resistance is tightly genetically linked with undesirable traits.

The development of gene technology has drastically increased the availability of genes conferring resistance, since they can now be derived from non-related plant species as well as non-plant sources. Additionally, the problem with co-transfer of linked undesirable characters from the gene source, as is often the case in traditional cross-breeding, can be avoided. Gene technology in combination with the increasing knowledge of various plant defense mechanisms contributes with new possible strategies that might be used for improvement of resistance.

Genes that provide resistance against plant diseases such as viruses, bacterial and fungal pathogens have been successfully introduced into crop plants. A recent example is transgenic papaya, which is resistant to Papaya ring spot virus (PRSV), and it is widely planted in Hawaii. It has helped to save the papaya industry from devastation by PRSV in 1999 (Gonsalves, 2004). No such commercial transgenic plant against bacterial or fungal pathogens is present now but research is still going on.

1.2. Plant Defense Mechanisms

Frequently, plants are exposed to impending pathogens. Although, most encounters result in resistance by the plant and uncommonly the result is disease. Most pathogens are much specialized and only influence a limited range of host plants, often only a single species. This uniqueness is due to the need for certain host factors for host recognition and to the plant's defense system.

1.2.1. Plant-pathogen interactions

The first line of defense includes constitutive barriers such as wax layers like cuticle, cell walls and chemical defense compounds. These barriers are present in the plant prior to any attack by pathogens and confer a broad resistance to a wide variety of parasites (Takken & Joosten 2000). If the first line of defense

is overcome a second line of induced responses may stop the invader. Further parasite attack on plants can cause either a compatible response with a successful infection of the plant or an incompatible response where the plant becomes resistant to the parasite. In compatible interactions, the parasite is often recognized too late and the outcome will be a diseased plant. In the case of an incompatible interaction, the plant rapidly recognizes the parasite and induces resistance mechanisms, which act very effectively against the invader (Métraux, 2001).

Induced responses to viruses, fungi, bacteria and certain insects generally require a specific interaction between plant and pathogen, which is described by the 'gene-for-gene' hypothesis (Flor, 1971). For a plant to be resistant to a specific pathogen race the plant must carry a resistance (*R*) gene that corresponds to an avirulence (*Avr*) gene of the pathogen. Briefly, the *R*-genes encode receptors for *Avr*-specified ligands, and by a direct or indirect interaction between *R*-gene and *Avr*-gene products the induced defense response is triggered.

The induced resistance response to pathogens can be divided into a local response at the site of infection and a systemic response in remotely located, yet unaffected plant parts. This induced systemic response is referred to as systemic acquired resistance (SAR) (Métraux *et al.*, 2002).

The local response includes modifications of the cell wall composition in surrounding cells as well as synthesis of antimicrobial compounds such as phytoalexins and pathogenesis-related (PR) proteins. Another feature of the primary, local response is the activation of a controlled process of cell death, which is called the hypersensitive reaction (HR) and will be considered in detail later on (Heil & Bostock, 2002).

The initial local defense response is followed by a signal spreading throughout the plant that induces changes in gene expression in uninfected distant

parts of the plant. This systemic response involves the onset of PR protein production and sometimes also phytoalexin synthesis (Heil & Bostock, 2002). The systemic signal can also alert the remote tissue to a faster defense response after challenging the infection, for example faster induction of cell wall lignification (Métraux *et al.*, 2002). SAR is regarded to be active against the major pathogen groups; viruses, fungi and bacteria, and is conferring resistance not only to the pathogen initiating the response but also to other pathogens. This resistance is rather non-specific and long lasting (Heil & Bostock, 2002). SAR has been demonstrated in over 30 plant species belonging to both mono- and dicotyledonous plant families (Métraux *et al.*, 2002).

PR proteins induced by SAR can be divided into many different families (Christensen *et al.*, 2002), where the function still is unknown for most of them. Some families contain proteins with chitinase or β-1,3-glucanase activity. These proteins hydrolyze chitin and β-1,3-glucan, respectively, which are important components of many fungal cell walls. Chitinases and β-1,3-glucanases have also been shown to possess antifungal activity *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993).

In contrast to these, plants must have the following properties to protect themselves from pathogen attacks:

1. A plant must not give the opportunity of being recognized by pathogens as a host.
2. A plant must have protections against the pathogens infiltration
3. A plant must have proper systems to prevent the reproduction of infiltrated pathogens and, even if it is affected by pathogens, minimize the probability of being killed by them.

1.2.2. Signaling Pathways

During plant-pathogen interaction, the recognition of an invading pathogen activates a signal transduction pathway that results in rapid induction of many plant defense mechanisms.

Salicylic acid (SA) is a key component of the signal transduction pathway leading to SAR. The levels of SA increase both locally and systemically after an infection event and the SAR induction is preceded by an increase of SA in the phloem. The SAR signaling pathway is not a linear chain of events but rather a complex network. Several pathways cooperate and then cause defense responses to target at various pathogens (Métraux *et al.,* 2002). One distinct branching point occurs downstream of SA with one branch related to PR protein production involved in resistance to fungal and bacterial pathogens and another branch conferring resistance to viruses. Depending on virus, virus-specific branch includes different mechanisms for virus resistance such as inhibition of replication, inhibition of cell-to-cell movement and inhibition of long-distance movement (Murphy *et al.,* 2001).

While SA-dependent SAR is generally induced by and active against various pathogens, a wound response is active mainly against herbivores. This response is induced by insect feeding and wounding, and results in a local defense reaction at the wound site as well as systemic responses that are transmitted throughout the plant. The defense response can be divided into direct and indirect responses. The direct responses include the production of anti-nutritive and toxic compounds such as proteinase inhibitors, polyphenol oxidases, alkaloids, terpenoids and phenolics as well as the formation of physical barriers. The indirect responses are constituted by the production of volatile compounds that attract predators and parasitoids of the attacking insects and repel herbivorous insects (Kessler & Baldwin, 2002).

Jasmonic acid (JA) is a central signaling molecule of induced plant responses to herbivores. Insect feeding or mechanical wounding is triggering the induced response where both oligosaccharides and oligogalacturonides released from damaged cell walls. The second step of the cascade is the release of linolenic acid from membrane lipids, which is then enzymatically converted to JA. Genes encoding proteinase inhibitors and enzymes involved in production of volatile compounds or secondary compounds, such as nicotine and numerous phenolics, as well as other defense-related compounds are transcriptionally activated by JA (Heil & Bostock, 2002).

1.3. Transgenic Strategies against Diseases

Although plants develop defense measures against pathogens, including both constitutive and induced defenses, they are still not always able to combat the invader. Even though new plant defense responses have evolved, like new R-genes, there is also a constant evolution of the parasites so that defense mechanisms may be overcome. One advantage of gene technology is that new defense factors which the parasites have not encountered before and thus not started to adapt yet, can be introduced into plants.

1.3.1. Strategies against viral diseases

The strategies for conferring virus resistance in plants by genetic engineering can be divided into two groups. The first group includes various strategies in which a plant is transformed with a gene, or part of a gene, from the virus against which resistance is desired. This strategy is named pathogen-derived resistance and can normally be explained by the virus-derived transgene interfering with essential steps in the life cycle of the infecting virus. The second group includes various strategies such as host resistance genes and other genes involved in defense responses that do not include viral genes

1.3.1.1. Studies regarding Pathogen-derived Resistance

Pathogen-derived resistance can be subdivided into different groups depending on which viral gene is involved. The most commonly used genes are coat protein genes, replicase genes and movement protein genes.

The first example of virus resistant transgenic plants was published by Powell-Abel *et al.* (1986) describing tobacco plants transformed with the coat protein gene of Tobacco mosaic virus (TMV) displaying improved resistance against TMV. Seedlings of transgenic plants that expressed the coat protein demonstrated significant delay in symptom development when inoculated with TMV.

Since this first demonstration, coat protein strategy has been extensively used for resistance against numerous viruses belonging to different groups in many crop plants as well as ornamentals (Liao *et al.*, 2004; Yang *et al.*, 2004). The approach of coat protein-mediated resistance has also been shown to be commercially applicable and a famous example of this, transgenic papaya, which is resistant to Papaya ring spot virus (Gonsalves, 2004).

Another type of gene used for conferring pathogen derived resistance is genes encoding viral replicase proteins. This technique is firstly developed against TMV (Golemboski *et al.,* 1990). Resistance mediated by replicase genes often confers a high level of protection but is generally only effective against viruses closely related to the source of the transgene. The efficiency of this strategy has also been confirmed in field trials and a commercial product combining resistance to the Polerovirus Potato leaf roll virus with resistance to Colorado potato beetle in transgenic Russet Burbank potatoes has been developed (Lawson *et al.*, 2001).

Plant viruses encode movement proteins that are required for viral cell-to-cell transport. These proteins contain nucleic acid binding domains and localize to

plasmodesmata where they facilitate the viral transfer to adjacent cells. One way to generate disease resistance is the transgenic expression of malfunctional movement proteins resistance to plant viruses can be achieved. The resistance was shown to be efficient not only to the homologous virus and related viruses but also to viruses belonging to other virus groups (Tacke *et al.*, 1996).

Some strains of certain RNA viruses contain satellite RNAs which can be used as another strategy for viral resistance. They are small RNA sequences that are fully dependent on the host virus for replication and transmission. The presence of satellite. Transgenic expression of suppressing satellite RNA sequences may attenuate symptoms when plants are infected with the host virus and it has been shown for Cauliflower mosaic virus in tobacco and pepper (Baulcombe *et al.*, 1986; Kim *et al.*, 1997).

Another strategy is the use of antisense RNA technologies. The expression of antisense RNA complementary to viral sequences has been used as a way to suppress expression of certain genes from the invading virus and thus improving resistance. This RNA-silencing strategy has been tested with antisense expression of such as coat protein and replicase genes (Cuozzo *et al.*, 1988; Powell *et al.*, 1989), but in some instances the resistance was only efficient at low inoculum concentrations.

1.3.1.2. Host resistance gene mediated studies

An alternative to use pathogen derived genes for introduction of resistance is to transform resistance (*R*) genes conferring virus resistance into heterologous plant species or varieties. The *N* gene of tobacco confers resistance to TMV by localizing the viral infection by a hypersensitive response (HR). When the *N* gene was transferred to tomato the resulting transgenic tomato plants showed typical HR as well as blocked systemic movement of the virus when the plants were challenged with the virus (Whitham *et al.*, 1996). In a similar way, also other *R*-

genes, such as the potato *Rx* gene conferring resistance to Potato virus X, have been shown to be functional when transferred to tobacco (Bendahmane *et al.*, 1999)

Another strategy about host resistance is ribosome-inactivating proteins, which have antiviral properties. A protein of this type from pokeweed (*Phytolacca americana*) was expressed in tobacco and potato, and was shown to confer broad-spectrum virus resistance both when inoculated mechanically and by aphids (Lodge *et al.*, 1993). However these proteins may also be toxic to the transgenic host plant. Therefore, less toxic forms of ribosome inactivating proteins have been used that still confers virus resistance but without affecting the host ribosome's (Zoubenko *et al.*, 2000).

Production of particular protein inhibitors also result in disease resistance. By expression of cysteine proteinase inhibitors in transgenic plants, virus replication and propagation can be blocked. In transgenic tobacco plants expressing oryzacystatin, a cysteine proteinase inhibitor from rice, there was a clear positive correlation between resistance to potyviruses and expression of oryzacystatin (Gutierrez-Campos *et al.*, 1999).

The expression of antibodies, so called plantibodies, which recognize plant viruses, is another approach for conferring virus resistance in transgenic plants. Single chain variable regions of monoclonal antibodies raised against a virus have been expressed in transgenic plants, which upon challenge with the corresponding virus demonstrated delayed symptom development and reduced incidence of infection (Tavladoraki *et al.*, 1993).

Interferons are mammalian proteins inducing various defense mechanisms that ultimately inhibit viral replication. One of these defense reactions is the 2-5A system, where 2'-5' oligoadenylate synthetase (2-5 Aase) makes 2'-5' oligoadenylates (2-5A) in response to double stranded viral RNA. The formed

2-5A then activates ribonuclease L (RNAseL), which degrade RNAs. Transgenic potato plants constitutively expressing 2-5 Aase have been shown to be protected against Potato virus X infection, also under field conditions (Truve *et al.*, 1993).

1.3.2. Strategies against bacterial and fungal diseases

While there are many successful examples of transgenic resistance to viruses, transgenic resistance to fungi and bacteria has generally been more difficult to accomplish and so far there are no commercial applications. The first efforts included the expression of single proteins, such as pathogenesis related proteins and other proteins and peptides with antimicrobial effects. More recently, due to the increasing knowledge about genes involved in plant defense pathways, the utilization of more complex defense responses has become possible.

1.3.2.1. Transformation of antimicrobial genes

As a general strategy, peptides and proteins with antimicrobial activities have been evaluated as transgenic resistance factors. Some plant-derived antimicrobial peptides, for example puroindolines from wheat and a novel peptide from *Macadamia integrifolia* nuts, have demonstrated antifungal effects in transgenic plants (Kazan *et al.*, 2002). Antimicrobial peptides of animal origin have also been assayed in transgenic plants. When the sarcotoxin gene from flesh fly was expressed in transgenic tobacco enhanced resistance against both bacterial and fungal pathogens were obtained (Mitsuhara *et al.*, 2000). In addition to naturally occurring antimicrobial peptides, also synthetic peptides have been designed and expressed in transgenic plants conferring improved resistance to bacterial and fungal pathogens (Cary *et al.*, 2000).

An example of proteins with antimicrobial activities are the ribosome inactivating proteins, that have been shown to confer resistance to fungal infection by inactivating foreign ribosomes (Logemann *et al.*, 1992). Other examples of

antimicrobial proteins are the antifungal proteins derived from a virus infecting *Ustilago maydis* (Clausen *et al.*, 2000) and from *Aspergillus giganteus* (Coca *et al.*, 2004) that have shown potential as transgenic resistance factors against fungal infection. Also antimicrobial proteins of animal origin, such as human lysozyme, have been demonstrated to improve bacterial and fungal resistance when expressed in transgenic plants (Nakajima et al., 1997).

Another possible target for transgenic resistance against fungal pathogens is the inactivation of pathogen produced enzymes and toxins which are needed for infection. During infection, fungal pathogens often produce plant cell wall degrading enzymes, such as polygalacturonase, as well as various toxins, such as oxalic acid and mycotoxins. The expression of a polygalacturonase inhibitor protein in tomato was able to reduce Gray-mold rot disease which is caused by *Botrytis cinerea* (Powell *et al.*, 2000).

1.3.2.2. Over-expression of plant defense related genes

Two pathogenesis related proteins, chitinase and β-1,3-glucanase have been extensively studied in transgenic plants. Firstly, it was demonstrated that heterologous expression of a bean chitinase in oilseed rape and tobacco could reduce the susceptibility to *Rhizoctonia solani* (Broglie *et al.,* 1991). Since then chitinases of various classes from different plant species and other sources, have been expressed in a large number of crops with effects against various fungal pathogens (Pappinen *et al.*, 2002). The effects demonstrated have mainly been a reduction of fungal development and reduced number and size of lesions. Also, partial resistance to fungal infection has been demonstrated in field trials (Grison *et al.*, 1996). When chitinase and β-1,3-glucanase enzymes are combined, they can act synergistically with improved suppressive effects on fungal infection. Transgenic tobacco has shown improved disease resistance when barley chitinase and β-1,3-glucanase were simultaneously expressed in tobacco while the resistance was stronger than their individual expressions (Jach *et al.,* 1995).

Also expression of pathogenesis related (PR) proteins from other classes, such as osmotin and thaumatin-like proteins (PR-5), in transgenic plants have been shown to have negative effects on fungal infections such as delayed development of disease symptoms (Liu et al., 1994; Datta et al., 1999). The cysteine-rich peptides such as defensins (PR-12), thionins (PR-13) and lipid transfer proteins (PR-14) are thought to be involved in induced defense responses and are also often found in seeds (van Loon & van Strien, 1999). Various such cysteine-rich peptides from different plant species have been expressed in transgenic plants where they have been shown to confer enhanced resistance to infections caused by fungi and bacteria (Gao *et al.,* 2000; Iwai *et al.,* 2002).

One more possible way to generate transgenic resistance plants against diseases regard phytoalexins which act as toxins to the attacking organism and may puncture the cell wall, delay maturation, disrupt metabolism or prevent reproduction of the pathogen in question. They are normally synthesized via complex biochemical pathways and are thus more complicated to produce transgenically. However, expression of some enzymes involved in phytoalexin synthesis, such as isoflavone-O-methyltransferase, demonstrates the potential of phytoalexins in conferring enhanced resistance to fungal infections (He & Dixon, 2000).

1.3.2.3. Transfer of resistance genes

R-gene transfer between related species has been shown to be another possible way to introduce disease resistance against bacterial and fungal pathogens. The *Pto* gene of tomato and *Bs2* gene of pepper have been transferred to other *Solanaceous* species conferring resistance to the bacterial pathogens *Pseudomonas syringae* and *Xanthomonas campestris*, respectively (Rommens *et al.,* 1995; Tai *et al.,* 1999).

Correspondingly, resistance to the fungus causing apple scab was introduced into a susceptible apple cultivar by transformation with the *HcrVf2* gene from the wild species *Malus floribunda* 821 (Belfanti *et al.,* 2004). Successful examples of transfer of R-genes have normally taken place between related species. However, for the *RPW8* genes of *Arabidopsis thaliana,* it has been shown that these are functional against powdery mildew also when transferred to tobacco (Xiao *et al.,* 2003).

A possibly more broad-spectrum approach to make use of *R*-genes and the SAR-pathway has also been described. According to this approach transgenic plants are transformed with pathogen *Avr*-genes under control of a heterologous infection-inducible promoter. If the generated transgenic plant carries the corresponding *R*-gene, a defense response will be initiated upon infection. One example in this direction is the pathogen-inducible expression in tobacco of the elicitor named cryptogein from *Phytophthora cryptogea*, a likely avirulence factor of Phytophthora spp. (Keller *et al.,* 1999).

1.3.2.4. Defense Signaling related studies

Different steps in plant defense pathways have been evaluated to get a better understanding of defense responses and potentially produce transgenic plants with improved defense systems. One step in the defense-signaling cascade that has been evaluated is the *NPR1* gene from Arabidopsis, which regulates salicylic acid signaling (Chern *et al.*, 2001). When *NPR1* was overexpressed in *Arabidopsis* as well as in rice, plants with stronger PR protein induction and enhanced bacterial and fungal resistance were generated. When bacterial SA-generating enzymes were expressed in transgenic tobacco, SA accumulation was substantially increased and PR proteins were constitutively expressed conferring enhanced resistance to fungal, as well as viral, infections (Verberne *et al.*, 2000).

Also expression of a transcriptional regulatory protein gene, *Tsi1* from tobacco, induced constitutive expression of PR proteins and conferred broad-spectrum resistance to both bacterial and viral diseases (Shin *et al.*, 2002). When the *Prf* gene which is involved in resistance to *Pseudomonas syringae* in tomato, was overexpressed in tomato, the transgenic plants displayed constitutively activated SAR with enhanced SA accumulation, constitutive PR protein synthesis and broad-spectrum resistance to bacterial and viral pathogens (Oldroyd & Staskawicz, 1998).

Another defense pathway step that has been modified is the expression of enzymes that generates reactive oxygen species. ROS play role in defense signaling and stimulate the plant resistance systems. Plants transformed with glucose oxidase genes that generate hydrogen peroxide, displayed enhanced tolerance to a broad spectrum of bacteria and fungi but also distorted plant growth (Wu *et al.,* 1995). Careful regulation of the glucose oxidase expression by pathogen-inducible promoters may overcome these negative effects (Kachroo et al., 2003).

1.3.2.5. RNA interference and Plantibodies

Gene silencing by RNA interference has been applied as a method to confer improved tolerance against the crown gall-causing bacterium *Agrobacterium tumefaciens*. Transformation of *Arabidopsis* and tomato by inverted repeats of the bacterial oncogenes resulted in plants that could still be infected by *A. tumefaciens* but where the formation of crown galls was completely prevented (Escobar *et al.,* 2001).

As for virus resistance, expression of antibodies which can bind to pathogen or pathogen products has been proposed as a strategy for conferring resistance to fungi and bacteria. However, so far the examples are very limited. Peschen *et al.* (2004) have expressed fusion proteins comprising chicken-derived single-chain

antibody fragments against *Fusarium graminearum* linked to antifungal peptides in *Arabidopsis thaliana*. When the transgenic plants were challenged with *Fusarium oxysporum* a high level of protection was obtained, whereas expression of either antibody or antifungal peptides alone resulted in moderate levels of protection only.

1.4. Hypersensitive Response

'Hypersensitive' was a term first used by Stakman (1915) to describe the rapid and localized plant cell death induced by rust fungi in rust-resistant cereals. The subsequent realization that such death was a common expression of disease resistance in plants, regardless of the type of inducing pathogen, led to its designation as the hypersensitive response, usually defined as 'the rapid death of plant cells in association with the restriction of pathogen growth'. The dead cells surrounding the infection site also may serve as a sink for deposition of antimicrobial compounds that are produced in response to pathogen infection. As a common sense, it is thought that inducing an HR during pathogen infection in plants may increase their resistance to diseases. Transgenic plants can be generated by transforming genes which control HR or which their products induce HR in cells, so that an overall disease resistance can be generated.

1.4.1. Genetic Control of HR

Recognition of the pathogen by plant cells is traditionally described by the ''gene-for-gene'' model (Flor, 1971). In this model, an avirulence factor encoded, or produced, by an *Avr* gene in the pathogen can be specifically recognized by a resistance (*R*) gene product in the host plant. When a pathogen carrying a particular *Avr* gene invades a host plant carrying the corresponding *R* gene, its recognition triggers the rapid activation of defense mechanisms and the proliferation of the microbe within the plant is inhibited. In the absence of either the *Avr* or its corresponding *R* gene, the pathogen is apparently not recognized by

the host and can proliferate within the plant leading to the appearance of disease symptoms (Keen, 1990).

It is indicated that there is such a direct interaction between the tomato *Pto* resistance gene product and the product of the avirulence gene *avrPto* from *Pseudomonas syringae* pv *tomato* (Tang *et al,* 1997). The analysis of the sequences of the different cloned resistance genes suggests that this possible type of direct interaction may not only happen in the plasma membrane but also in the cytoplasm and in the nucleus (Van den Ackerveken *et al.,* 1996)

R genes are often allelic, exist in clusters in the plant genome, and have been suggested to resemble the vertebrate major histocompatibility complex in organization and evolution (Michelmore and Meyers, 1998). Cloned *R* genes share common themes and plant genomes appear to contain large numbers of genes with similar sequences. Most of their predicted proteins have leucine-rich repeats (LRRs) or a serine-threonine kinase domain and are assumed to be components of signaling systems as one might expect of molecules that detect a specific pathogen's presence. The predominant class of predicted *R* gene products has a nucleotide-binding site (NB) as well as LRRs (Heath, 2000).

Although *R* genes were the first genes to be identified by Mendelian genetics to control the HR in resistant plants, mutation studies have revealed that the HR also depends on additional genes that presumably are present in both resistant and susceptible members of host species and which confer the ability of all plants to undergo an HR even in non-gene-for-gene situations (Heath, 2000). These RDR (required for disease resistance) genes may be different for different *R* genes, irrespective of the type of pathogen against which they act (Morel and Dangl, 1999).

1.4.2. Elicitors involved in HR

In addition to *Avr* gene products, pathogens have a variety of components or secretory products, such as arachidonic acid, cell wall carbohydrates, glycoproteins and proteins, that can elicit plant defense responses and, in some cases, cell death (Chen and Heath, 1994). There is no requirement for the presence of a living pathogen to trigger the HR and certain purified elicitors can induce many of the physiological changes occurring during disease resistance and lesions resembling the HR (He et al, 1993)

One of these elicitors, harpin, induces HR in non-host plants of bacteria. They are glycine-rich, protease-sensitive, heat-stable, acidic proteins produced by Gram-negative pathogenic bacteria and are governed by the *hrp* (HR and pathogenicity) gene cluster (Bonas, 1994). Application of harpin to various plant species enhances plant growth, and induces resistance to pathogens and insects (Kim and Beer, 2000). These effects were found first in plants treated with harpin from *Erwinia amylovora*, the first reported bacterial cell-free HR elicitor (Wei *et al.,* 1992).

Three genera of plant bacterial pathogens, *Erwinia, Pseudomonas,* and *Ralstonia* export harpins via the type III protein secretion system. It has been suggested, but not yet proven, that harpins perform various functions by activating distinct signaling pathways. Genetic evidence indicates that harpins may play a minor role in bacterial elicitation of the HR, but it may assist the delivery of other pathogenesis proteins across the plant cell wall (Galan and Collmer, 1999). HR induced by harpin from *Erwinia amylovora* or *Pseudomonas syringae* pv. *syringa*e was prevented by inhibition of calcium influx and ATPase activity in tobacco cell suspensions (He *et al.,* 1993). Harpin has a pronounced effect on the plasmalemma, affecting H^+-ATPase, ion channels or membrane carriers. These results indicate that the site of harpin action is the plant cell membrane and cell wall (Pike *et al.,* 1998)

1.4.3. Signal Transduction pathways leading to HR

Some of the early events associated with the HR involve rapid generation of reactive oxygen species (ROS), the so-called oxidative burst (Tenhaken *et al.,* 1995), and a rapid flux of ions across the plasma membrane, the so-called XR (Nurnberger *et al.,* 1994). Although the role of the XR in inducing the HR is not clear, ROS have direct antimicrobial activities and can therefore reduce pathogen viability. They have also been implicated in the destruction of the challenged plant cells, either through lipid peroxidation or function as a key factor mediating programmed cell death (Bethke and Jones,2001).

H_2O_2, the most stable ROS, functions as a local trigger for the programmed death of challenged cells and as a diffusible signal to induce resistance genes in surrounding cells (Levine *et al.*, 1994). Furthermore, H_2O_2 plays a role in the phenomenon of cross-tolerance, in which exposure to one stress can induce tolerance to other stresses (Bowler and Fluhr, 2000). Several possible sources of ROS in plants and a number of biotic and abiotic stresses can stimulate oxidative burst. In order to summarize, overall representation of the events taking place during HR process is given in figure 1.1

The signal transduction pathway that leads to the activation of the HR was also suggested to involve increases in Ca^{2+} flux and protein phosphorylation (Levine *et al.,* 1994). Plants commonly respond to external stimuli, including microbial elicitors of cell death and defense responses, by calcium influx into the cell. An increase in cytosolic calcium precedes, and seems necessary for, hypersensitive cell death triggered by rust fungi (Xu and Heath, 1998) and the calcium channel blocker La^{3+} prevents bacterial-induced HR in soybean leaves (Levine *et al*., 1994).

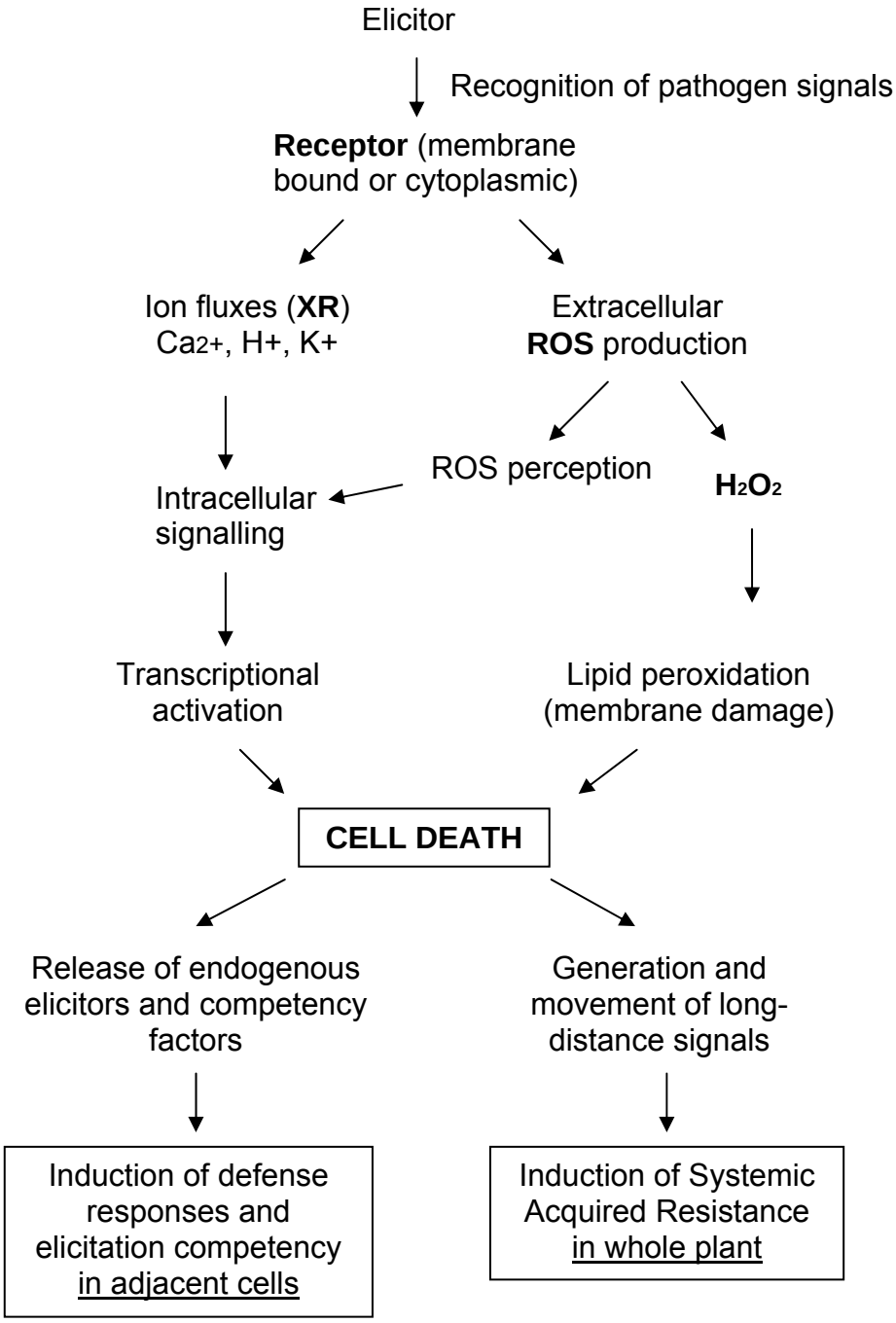

Figure 1.1 An overall representation of the events taking place during HR process. The defense responses are composed of defense gene activation (structural proteins, phytoalexins biosynthesis genes, anti-fungal proteins) and productions of endonucleases and proteases(Morel and Dang, 1999; Heath, 2000).

Although the signal transduction events that lead to the activation of HR and defense mechanisms during the HR are controlled by a single gene-for-gene interaction (*R* in the plant and *Avr* in the pathogen), it appears as if two distinct pathways may be set into motion by this recognition event. One pathway controls the activation of HR whereas the other controls the induction of PR proteins and perhaps other defense mechanisms.

1.4.4. Reactions of HR process

Unlike mammalian apoptosis, the reactions during plant programmed cell death and HR can not be generalized yet. The first observable sign of the HR occurs a few hours after the pathogen enters the cell although the induction of hypersensitive cell death depends on changes in cytosolic calcium levels (Mould *et al.*, 1999). Cell death becomes irreversible at the point where microtubules disappear and cytoplasmic streaming ceases, then the cell undergoes a prolonged disassembling of the protoplast which may take several hours and consecutively involves the appearance of particles showing Brownian motion in the plant vacuole, cleavage of nuclear DNA and the intracellular generation of hydrogen peroxide (Heath *et al*., 1997). Finally, the plasma membrane loses its semi-permeability and the protoplast shrinks in a manner suggestive of contraction rather than loss of turgor. The cell then becomes brown, most probably due to the accumulation and oxidation of phenolic compounds secreted by surrounding cells and granules of nucleic acid appear in the plant nucleus (Škalamera *et al.*, 1998).

During the death process in some forms of mammalian apoptosis, the release of cytochrome *c* from mitochondria causes the cascading activation of a family of cysteine proteases known as caspases that results in the degradation of a number of cell components including poly (ADP-ribose) polymerase (Green and Reed, 1998). Plant cells contain similar proteases. Data from D'Silva *et al*., (1998) and del Pozo and Lam (1998) suggest that the dismantling of the cell during the HR involves the activation of cysteine proteases as it does during mammalian

apoptosis. However, plants can produce a variety of proteolytic enzymes and in a virus system at least, it seems likely that other proteases are involved as they are in developmental forms of programmed cell death in plants and some forms of elicitor-induced cell death (Groover and Jones, 1999).

Another hallmark of apoptosis is the cleavage of nuclear DNA into oligonucleosomal fragments, producing a 'ladder' of DNA when run on agarose gels. Plant DNA cleavage has been reported for fungus-, bacteria-and virus-induced HR (Levine *et al*., 1996) but its timing during the death process varies and it results in a DNA ladder only during the fungus-induced response (Mittler *et al.,* 1997)

1.5. Plant ferredoxin-like protein (PFLP)

Recently, a plant ferredoxin-like protein was reported to increase the generation of harpin mediated ROS production and HR in tobacco suspension cells by Lin *et al.* (1997). It is thought that generating transgenic tobacco with *pflp* gene can confer resistance to various harpin producing pathogenic bacteria.

1.5.1. Structure of PFLP

A 662 bp full-length cDNA clone (Figure 1.2) encoding native PFLP protein was isolated from sweet pepper and characterized by Drakkar *et al.* in 2003. When *pflp* was sub-cloned to an *E.coli* vector and recombinant proteins was expressed, it had been understood that the recPFLP was same as native PFLP by SDS-PAGE analysis with Anti-PFLP polyclonal antibodies. The actual molecular weight of the native PFLP protein and recombinant PFLP protein in mass spectrophotometric analysis and based on amino acid sequence analysis was evaluated as 12.58 kDa.

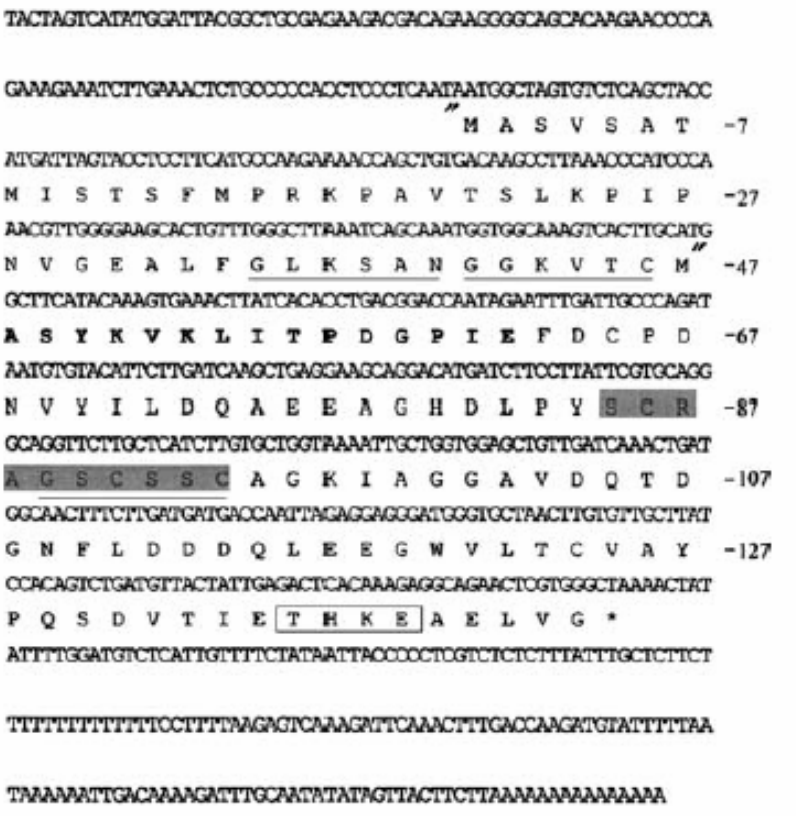

Figure 1.2 Nucleotide and deduced amino acid sequence of *pflp* cDNA from sweet pepper. The 144 deduced amino acid sequence indicates the 432 bp open reading frame. The shaded region shows the putative 2Fe-2S domain. The box area indicates the casein kinase II phosphorylation site. Underlined area indicates the N-myristoylation site. The stop codon is indicated by ∗. “ ” indicates a putative signal peptide region (Dayakar *et al.*, 2003).

Sequence comparison in database searches suggested that the PFLP protein is a ferredoxin-I containing a Ser- and Thr-rich N-terminal signal peptide of 47 amino acids targeting to chloroplast and putative 2Fe-2S domain. The PFLP protein sequence showed identity to *Lycopersicon esculentum* ferredoxin-I (72%), pea ferredoxin-I (52%), *Arabidopsis thaliana* ferredoxin A (54%), *Spinacia oleracea* ferredoxin-I (52%), *Oryza sativa* ferredoxin (56%) and maize ferredoxin

(48%). These ferredoxins are thought to be electron carriers in photosynthetic tissues. Among these sequence comparisons, it showed maximum positional identity with *L. esculentum* ferredoxin-I. Sequence comparison showed the highest amino acid homology with ferredoxin-I of *L. esculentum*, sharing an identity of 72% and a similarity of 81% (Dayakar *et al.,* 2003).

1.5.2. Function of PFLP in HR

The function of PFLP in HR is not yet clear but it is suggested that PFLP may interfere with the interaction between harpin and a putative receptor, and also with the *Avr* gene/R-gene mediated HR induction (Keen, 1990). In one study, inhibition of the harpin-mediated HR by PFLP was shown to be dosage-dependent and revealed a competitive pattern. PFLP may interact with harpin as a putative receptor so as to prevent the binding between receptor and the active fragment of harpin. In this way, harpin may retain its ability to activate an HR via the signal transduction system (Lin *et al.,* 1997).

The production of reactive oxygen species (ROS) is one of the earliest events during HR and considered as a characteristic of plant defense responses (Levine *et al.,* 1994) Moreover, PFLP protein is a ferredoxin that is involved in many redox reactions leading to the production of ROS and it is found that PFLP enhances ROS production, so as a result intensifies the harpin-mediated HR (Dayakar *et al.,* 2003).

1.5.3. Transformation studies with PFLP

Several transformation studies were conducted with *pflp* in different plant species. Initially, transgenic rice expressing PFLP was produced by Tang *et al.* (2001). It is thought to raise resistance against one of the most devastating diseases of rice in Africa and Asia, bacterial leaf blight, which is caused by the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Indeed,

transgenes show resistance to *Xoo* race 6 but not to all races. Likewise in another study, transgenic plants over-expressing a rice resistance gene *Xa21*, show enhanced resistance to bacterial blight caused by most *Xoo* races but not all (Wang *et al.,* 1996). There is always the danger that susceptible pathogens will evolve resistance to the transgenes used against them. So the resistance genes and defense stimulating genes can be used in combination and their effect would be cumulative.

Another study shows that *pflp* confers resistance against soft rot disease which is caused by *Erwinia carotovora* in *Oncidium* orchid even when the entire plant was challenged with the pathogen (Liau *et al.,* 2003). There has been an enormous effort to improve ornamental plant traits such as disease resistance and this study is one of the first to introduce resistance to diseases. One more importance of this study was being the first successful production of transgenic orchids using *Agrobacterium* –mediated transformation. A different study reveals that *pflp* can be used as a novel selection marker in genetic transformation of *Oncidium* orchid (You *et al.,* 2003). *E. carotovora* was used as a selection reagent to screen transformants without the use of an antibiotic agent as *pflp* confers resistance to this pathogen. However, using other crop plants which are not natural hosts of *E. carotovora* as transformation materials might be the limitation of this selection system.

1.6. Aim of the study

The objective of the present study was to assess the influence of *pflp* gene expression in transgenic tobacco (*Nicotiana tabacum* cv. Samsun) plants and develop PFLP mediated hypersensitive response for various harpin producing pathogens.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains and plasmid

The supervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood, 1993) containing pSPFLP was used in plant transformation studies. A DNA fragment, containing a cauliflower mosaic virus (CaMV) 35S promoter, a *pflp* coding sequence (including the signal peptide) and a nopaline synthase (*nos*) poly(A), was excised from pBIAP1 by digestion with *Eco*RI and *Hind*III (Tang *et al.* 2001) and cloned into the *Eco*RI and *Hind*III site of pCAMBIA 1304 to form pSPFLP (Appendix A). The bacteria were donated kindly by Dr. Ming-Tsair Chan, from Institute of BioAgricultural Sciences, Academica Sinica, Taipei, Taiwan, Republic of China.

Pseudomonas syringae pv. *syringae* and *Pseudomonas syringae* pv. *pisi* were used for Hypersensitivity Response (HR) assays and was kindly donated by Dr. Fikrettin Şahin, Atatürk Üniversitesi, Erzurum, Turkey.

2.1.2. Bacterial Culture Media and Culture Conditions

Nutrient broth, yeast extract, sucrose and magnesium sulphate containing Yeast Extract Broth (YEB) was used to grow *A. tumefaciens* EHA105 strain. Also

the medium was supplemented with 20 mg/L Rifampicin and 50 mg/L Kanamycin for bacterial selection.

Also, for *Pseudomonas syringae* strains, Yeast Extract Broth (YEB) medium was preferred. The composition of bacterial culture medium is provided in Appendix B.

All the bacterial cultures were grown with a 180-200 rpm (revolution per minute) in a Sanyo Gallenkamp shaker at 28°C. When needed 1.5% agar was added to solidify the media. A liquid aliquot of each bacterial strain was kept at -80^0C in 20% glycerol and at -20°C in 50% glycerol for longer storage.

2.1.3. Plant Material and Tissue Culture Media

Throughout the tissue culture and transformation studies of this work, seeds and leaves of *Nicotiana tabacum* L. cultivar Samsun were used as the explant source.

MS (Murashige and Skoog, 1962) based media containing MS micro-macro elements and vitamins with additions of sucrose and agar were used for all plant tissue culture media. The composition of the media is presented in Appendix C.

The entire medium was dissolved in distilled water, and then pH of the medium was adjusted to 5.7-5.8 with NaOH. Finally the medium was sterilized by autoclaving at 121^0C for 15 minutes. Filter sterilized plant growth regulators and antibiotics (naphtalenaceticacid [NAA], benzylaminopurine [BA], cefotaxime, rifampicin, and kanamycin) were added freshly to the sterile medium. The composition and the purpose of the media are given in Table 2.1.

Table 2.1 The composition and the purpose of plant tissue culture media

Media	Composition	Purpose of Use
Liquid MS	M5519 + 30 g/L sucrose	Diluting *Agrobacterium* culture before transformation
MSA	M5519 + 30 g/L sucrose + 0.8% plant agar + 1 mg/L BA + 0.1 mg/L NAA	Callus and shoot formation for control leaf discs, co-cultivation for *Agrobacterium* treated leaf discs
MSB	MSA + 25 mg/L Hygromycin + 500 mg/L Cefotaxime	Selection of transformed leaf discs and their regeneration
MSC	MSA (for [+]control shoots) or MSB (for [-] control and transgenic shoots) : without plant growth regulators	Induction of root formation on regenerated shoots, sub-culturing and germination of the surface sterilized seeds

2.1.4. Plant Tissue Culture Conditions

All plant tissue cultures were incubated at 24±2ºC under fluorescent light at 100 µmol $m^{-2}s^{-1}$ with a 16/8 hour (light/dark) photoperiod.

2.1.5. Other Chemicals and Materials

The chemicals used in this study were purchased from Sigma Chemical Company (N.Y., USA), Merck Chemical Company (Deisenhofen, Deutschland), Applichem (Darmstadt, Germany), and Duchefa (Haarlem, The Netherlands). Other chemicals and enzymes used in molecular biology studies were from MBI Fermentas (Ontario, Canada) and Amersham Biosciences (NJ, USA). All of the media and solutions were prepared by using distilled water.

Glassware (autoclavable capped tissue culture jars) was from Sigma and Vitro Vent Containers (VVC) was from Duchefa. 0.2 μm minipore filters were from Sartorius.

2.2. Methods

2.2.1. Verification of *pflp* within the plasmids

In order to prove that *pflp* is present in the plasmids of *A. tumefaciens* cells, plasmid isolation and PCR studies were performed

2.2.1.1. Plasmid Isolation

With the purpose of using in further studies, the plasmid pSPFLP was isolated in pure form. For isolation a modified Mini Scale Plasmid Isolation protocol (Maniatis *et al*., 1989) was used.

A single colony of *A. tumefaciens* EHA105 pSPFLP was taken from the selective agar plate by loop. It was transferred to 3 mL of YEB medium containing 50 mg/L Kanamycin and 20 mg/L Rifampicin. The cultures were grown with vigorous shaking at 120 rpm and 28°C. Overnight grown bacterial cells were divided into eppendorf tubes with 1, 5 ml aliquots and centrifuged at 12000 g for 30 seconds in microcentrifuge.

The supernatant was discarded and the pellet was suspended in 1 ml of STE buffer (Appendix D). Again they were centrifuged at 12000 g for 1min. The supernatant was discarded and this time the pellet was suspended 100 μL of SOL I (Appendix D) with vigorous vortexing. Then they were incubated for 5 minutes at room temperature and 200 μL of SOL II (Appendix D) was added into each eppendorf tube. Slowly the tubes were mixed 4-5 times by inverting them upside

down and incubated 5 minutes at 60°C. 150 µL of cold SOL III (Appendix D) was added into the tubes gently, and the tubes were incubated in ice for 10 minutes.

Equal volume (450 µl) of chloroform:isoamylalcohol (24:1) was added to separate cell debris and chromosomal DNA in different phases and mixed by vortexing. The tubes were centrifuged at 12000 g for 5 minutes and upper DNA phase was collected into a clean eppendorf tube. Plasmid DNA was precipitated with addition of 2 volumes of cold 96% ethanol and incubation at least for 30 minutes at -20°C. Overnight incubation may be preferable. The plasmid DNA was obtained in the pellet after centrifugation at 12000 g for 5 minutes. Pellet was washed with 1ml of 70% ethanol, centrifuged at 12000 g for 2 minutes and ethanol was removed. The pellet was dried completely at 37°C and dissolved in 50 µL of TE buffer (Appendix D) containing 20 µg/mL RNase.

After the incubation for 30 minutes at 37°C for RNase activity, plasmid DNA was stored at –20°C until usage. Purity and integrity of DNA was confirmed by spectrophotometric measurement (OD_{260} and OD_{280}) and agarose gel electrophoresis, respectively.

2.2.1.2. PCR Analysis

The Polymerase Chain Reaction (PCR) was performed in order to verify the presence of *pflp* gene within the plasmids. For this purpose, the gene was amplified with specific primers (Appendix E).

PCR was carried out in a total volume of 30 µL. Cycling program of PCR amplification and optimized conditions of PCR are given in Table 2.2 and Table 2.3 respectively.

Table 2.2 PCR cycling conditions to amplify *pflp* gene for plasmid DNA

Initial denaturation	94°C 3min	
Denaturation	94°C 45 s	35 cycles
Annealing	62°C 30 s	
Extension	72°C 45 s	
Final extension	72°C 7min	

Table 2.3 Optimized conditions of PCR to amplify *pflp* for plasmid DNA

Reagent	**Volume**
10 X Rxn Buffer with $(NH_4)_2SO_4$	3 µl
25 mM $MgCl_2$	1.5 µl
2.5 mM dNTP	2 µl
10 µM Primer F	2 µl
10 µM Primer R	2 µl
dH_2O	18 µl
Taq	0,5 µl
Template DNA	1 µl
Total volume	30 µl

DNA molecules obtained throughout this study were visualized on agarose gels. Depending on the size of DNA to be detected 0.6 %, 0.8 % or 1 % agarose gels were used. For this purpose 0.3 g, 0.4 g or 0.5 g of agarose was dissolved in 50 mL of 0.5X TBE or 1X TAE buffer (Appendix E) in microwave oven, respectively. Without boiling, when it completely dissolved it was cooled down to approximately 50°C and 3 µL of ethidium bromide (so the final concentration will be 6 mg/mL) was added to gel solution. The comb was placed for well formation and without making bubbles the solution was poured into the electrophoresis tray. When the gel completely solidified, electrophoresis buffer was poured to the tank till it covers the gel for 5 mm and the comb was removed carefully.

The sample solution to be loaded was prepared by mixing samples with 6X loading buffer to a final concentration of 1X and loaded into the wells. A

molecular size marker, DNA ladder, was also loaded into a separate well. Then the tank was connected to a power supply and electrophoresis was performed under constant voltage of 6 V/cm for 1,5 - 2 hours. The gel was observed under UV light and photographed.

2.2.2. Maintenance of Plant Material

2.2.2.1. Surface Sterilization of Tobacco Seeds

The tobacco seeds were taken into an eppendorf tube and surface sterilized with 30% (v/v) sodium hypo chloride for 20 minutes and rinsed 3 times with sterile distilled water. The seeds were kept in sufficient amount of distilled water and placed on culture medium with the help of a cut micropipette.

2.2.2.2. Micropropagation of Tobacco Plants

The surface sterilized tobacco seeds were germinated on MSC in a growth chamber at 25±2^{o}C with 16 hours light and 8 hours dark photo- cycle for a month. The meristematic part of the emerging shoots were cut and placed into new jars containing the same medium called sub-culturing and used in further studies.

2.2.2.3. Determination of Lethal Hygromycin Concentration

pSPFLP contained *hph* (hygromycin phoshotransferase gene) in T-DNA region, which enables the putative transgenic plants to withstand the toxic effects of hygromycin where as wild type (control) ones can not.

First of all, in order to use negative controls in the experiments, the lethal hygromycin concentration for tobacco plants was determined. Leaf discs which were from 90-days old healthy upper leaves of wild type tobacco plants, were prepared and placed on M9274 medium (Appendix C) containing 0.1 mg/L NAA,

1 mg/L BA and varying concentrations of hygromycin which were 5 mg/L ,10 mg/L, 25mg/L ,50 mg/L and 100 mg/L . After 3-4 weeks they were photographed.

2.2.3. Leaf Disc Transformation of *Nicotiana tabacum*

A modified transformation method of Öktem *et al*. (1994) which was first described by Horsch *et al*. (1985) was used in the transformation studies of tobacco leaves.

Young leaves from 20-30 days old tobacco plants were used. Either sterile plant were used or non-sterile plants were first surface sterilized in 1.5% (v/v) Sodium Hypochloride and rinsed 3 times with distilled water then used in plant transformation studies.

Edges, midribs and petioles of the tobacco leaves were removed and the remaining parts were cut into 1-2 cm^2 pieces, which will be referred as "leaf disc" in the text. *A. tumefaciens* cells were grown overnight (OD_{600} = 0.6-0.8) in YEB medium containing appropriate antibiotics at 28^oC and they were diluted 1:10 times with liquid MS medium. Leaf discs were incubated in liquid MS and *Agrobacterium* cell mixture for 10 minutes. As a control, some discs were incubated in liquid MS only.

At the end of incubation period, the leaf discs were removed and the excess *Agrobacterium* solution remaining on the leaf discs were blotted on sterile filter paper. Then, the explants were transferred on MSA media and kept at dark for co-cultivation for 3 days. After the co-cultivation period, the explants were washed with liquid MS containing 750 mg/L cefotaxime for 3 hours with vigorous shaking at 120 rpm. The leaf discs were blot dried on sterile filter paper and transferred onto MSB media and kept at normal growth conditions. Plates were refreshed at every two weeks.

Callus formation was observed 3-4 weeks after the experiment. Regenerated shoots were cut from the attachment points to callus and transferred to jars containing MSC media to induce root formation and further development. When roots become stronger, regenerated plants were removed from jars and transferred to soil and transferred to growth chamber and then 4 weeks later to greenhouse. When flowers developed they were covered with nylon bags to prevent cross-pollination. When dried completely, about 3,5-4 months later, seeds of putative transgenic plants were collected.

2.2.4. Analysis of T_1 Transgenic Tobacco Plants

All assays and molecular tests were carried out with T_1 plants in order to prove that the transferred T-DNA is expressed in the plant genome. For this purpose, 5 lines were randomly selected from 15 different putative transgenic lines (T_0)

2.2.4.1. Mendelian Inheritance Pattern

Both putative transgenic tobacco seeds (T_1) and wild type tobacco seeds were used (as negative control) to confirm Mendelian inheritance pattern for T_1 seeds. They were surface sterilized as explained before and then they were placed on MSC medium (without Cefotaxime). For each line, nearly 100 seeds were used. They were incubated as stated before. At the end of six weeks the plates were photographed. These randomly selected hygromycin resistant lines, used for further studies, where as hygromycin sensitive lines were named as escape lines.

The number of surviving plantlets on hygromycin containing medium was counted after 6 weeks and then Chi-Square Analysis were performed to test the Mendelian Inheritance Pattern.

2.2.4.2. GUS Histochemical Assay

Histochemical GUS staining was performed according to the method of Jefferson (1987) in order to monitor the transient gene expression in T_1 leaves. Explants were assayed by incubating them inside 1 mM chromogenic substrate X-GlcA(5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) containing GUS substrate solution for overnight at 37°C. Then the explants were transferred to fixative solution in which they can be preserved for several months.

After a minimum of 4 hours in fixative, the solution was replaced with 50 % ethyl alcohol for decolorization of explants. After 15 minutes in 50 % ethyl alcohol, explants were transferred to 100 % ethyl alcohol for further decolorization overnight. Then the explants were transferred to GUS fixative solution for preservation for several months. Finally GUS expressing regions on explants were examined under microscope and photographed.

Preparation of GUS substrate solution and fixative solution were given in Appendix F.

2.2.4.3. Molecular Analysis of T_1 via PCR

2.2.4.3.1. DNA isolation from T_1 leaves

The genomic DNA was isolated from plants with CTAB DNA extraction method of Saghai-Maroof *et al*. (1984) with some modifications. According to this method, 2 grams of plant material was ground to a fine powder with a pestle and mortar in liquid nitrogen and transferred to centrifuge tubes. 2X CTAB buffer (Appendix G) was heated to 65^{o}C and 20 mL of 2X CTAB buffer ,containing 0.2% β-mercaptoethanol , was added onto the powder in a centrifuge tube and mixed by vortex. Then the 40 ml Sigma centrifuge tubes were incubated at 65^{o}C for at least 45 minutes with occasional shaking.

After incubation period, 20 mL of chloroform: isoamylalcohol (24:1) was added and the mixture was mixed vigorously by vortex. They were centrifuged at 3000 rpm for 10 minutes. The upper phase was taken to a new centrifuge tube and 8 mL of preheated 0.2% β-mercaptoethanol containing 5X CTAB buffer was added. Again the tubes were mixed by vortex and incubated at 65^{o}C for 10-15 minutes. Then 20 mL of chloroform was added to each tube and they were mixed by vortex. The tubes were centrifuged at 3000 rpm for 10 minutes.

Upper phase was taken to a new centrifuge tube and 20 mL of cold isopropanol was added into the tubes and the tubes were inverted gently to precipitate DNA. The tubes were kept overnight at –20^{o}C and, DNA was collected by centrifugation at 9000 rpm for 15 minutes. The upper phase was discarded and the pellet was dried with filter paper. The pellet was washed with 70% ethanol to get rid of the remaining impurities, centrifuged at 9000 rpm for 5 to 10 minutes and dried at 37^{o}C. Finally, the pellet was dissolved in 500 µL of TE buffer (Appendix D) containing 10 µg/mL RNAse. The tubes were incubated at 37^{o}C for 30 minutes for RNAse activity. The purity and concentration of DNA was checked by spectrophotometric measurement and agarose gel electrophoresis. The prepared DNA was stored at –20^{0}C for further usage.

2.2.4.3.2. PCR with *pflp* and *hph* specific primers

The isolated DNA from T_1 transgenic plants and control plant were analyzed with PCR for the presence of *pflp* and *hph* gene. The primers used in both reactions were given in Appendix E. Cycling program of PCR amplification and optimized conditions of PCR for *pflp* and *hph* from isolated DNA samples are given in Table 2.4 and Table 2.5, respectively. According to the DNA concentration of T_1 transgenic plants, the volume of 1 µg of DNA was determined.

Table 2.4. PCR cycling conditions to amplify *pflp* and *hph* for plant DNA

	pflp	*hph*	
Initial denaturation	95°C 4min	95°C 4min	
Denaturation	94°C 1min	94°C 50 s	
Annealing	62°C 1min	61°C 50 s	30 Cycles
Extension	72°C 1min	72°C 50 s	
Final extension	72°C 10min	72°C 7min	

Table 2.5. Optimized conditions of PCR to amplify *pflp* and *hph* for plant DNA

Reagent	*pflp*	*hph*
dH_2O	20-X µL	20-X µL
10X Rxn Buffer with $(NH_4)_2SO_4$	3 µL	3 µL
25 mM $MgCl_2$	1.5 µL	1.5 µL
2 mM dNTP	2 µL	2 µL
10 µM Primer Forward	1,5 µL	1,5 µL
10 µM Primer Reverse	1,5 µL	1,5 µL
Taq Polymerase (5 u/µL)	0.5 µL	0.5 µL
DNA (1 µg)	X µL	X µL
Total Volume	30 µL	30 µL

At the end of PCR, the products were run on 0.8% agarose gel and they were photographed under UV as described before.

2.2.4.3.3. Sequence Analysis of PCR products

The verification of the amplicons was also done with sequence analysis. For this purpose, the service of Sequence Analysis Unit of Central Laboratory, METU was used.

For this analysis, only line 6, which gave positive results for GUS histochemical staining and positive reactions for both PCRs with *pflp* and *hph* primers, was selected. 3 individual PCRs were carried out with primers of *pflp*, NOS terminator and CaMV 35S promoter genes (The primers, PCR and cycling conditions were given in Appendix H). About 100 µL from each PCR mixture were given directly to the Sequence Analysis Unit and according to the Unit the following procedure had been applied.

First, using Q-Biogene's GENECLEAN® *Turbo* for PCR Kit, the amplicons were purified from the PCR mixture as instructed on manual. 100 µl oil-free PCR reaction from each sample was transfered to a 1.5 ml microcentrifuge tube and 5 volumes GENECLEAN® *Turbo* for PCR Salt Solution was added. The tubes very gently mixed and transfered to a GENECLEAN® *Turbo* for PCR Cartridge. After centrifugation for 5 seconds, 500 µl prepared GENECLEAN® *Turbo* for PCR Wash was added to the filter. After another centrifugation for 5 seconds, catch tubes were emptied. The GENECLEAN® *Turbo* for PCR Cartridge were centrifuged for an additional 4 minutes to remove residual wash solution. Caps were removed from catch tube, and inserted to GENECLEAN® *Turbo* for PCR Cartridge containing bound DNA. Then, 30 µl GENECLEAN® *Turbo* for PCR Elution Solution was added directly onto GLASSMILK®-embedded membrane and incubated at room temperature for 5 minutes. Finally, centrifuge for 30 seconds was applied to transfer eluted DNA to GENECLEAN® *Turbo* for PCR Catch Tube. GENECLEAN® *Turbo* for PCR Cartridges were discarded and catch tubes were capped.

Then sequencing PCR was carried out by Applied Biosystems' BigDye® Terminator v3.1 Cycle Sequencing Kit according to the kit's manual and finally sequencing analysis was done with ABI 310 DNA Analyzer.

The results of sequencing analysis were investigated using Chromas (v2.23) software program. “BLAST search” option, which was built in the software, was used to compare the sequences with previously characterized genes.

2.2.4.3.4. Real Time-PCR Analysis

In addition to these, Real Time (RT) - PCR was carried out by the service of RT-PCR Unit of Central Lab., METU. According to the Unit, the following procedure had been applied.

First, Roche Applied Science’s High Pure GMO Sample Preparation Kit was used. The kit was designed for the isolation of DNA from raw material and food products of plant origin for PCR analysis. So it was used to isolate DNA from wild type control and T_1 lines of 1,3,6,9 and 10 as instructed on the kit’s manual.

After DNA isolation, RT-PCR was carried out by Roche Applied Science’s LightCycler ® GMO Screening Kit. The instrument used for RT-PCR was Roche Applied Science’s LightCycler ® 1.5. Finally, the results were evaluated by the instrument’s software.

2.2.4.4. Expression Analysis of *pflp*

Northern Blot Hybridization was used to show the *pflp* expression in T_1 transgenic tobacco plants using leaf samples of selected lines.

2.2.4.4.1. Total RNA Isolation

Prior to RNA isolation, distilled water, mortars, pestles, eppendorf tubes, tips and other equipments were washed with DEPC-treated water and placed under hood for overnight to evaporate the excess DEPC. All the solutions used in

the RNA isolation were prepared with DEPC-treated water and autoclaved before use. For 1 L of water 800 µL of DEPC was used.

Grinding step was crucial. Mortar, pestle and eppendorf tubes should be chilled with liquid nitrogen before usage. Quickly, 0.1 gram of leaf sample was ground in liquid nitrogen to obtain a fine powder. Eppendorf tubes were one-third filled (till 0, 5 ml line) with powder and transferred into liquid nitrogen and they were never let to be warmed up. 1 mL TRI reagent (Appendix I) was added and the eppendorf tubes were mixed by vortex at least 10 minutes. Then they were centrifuged for 5 min at room temperature with at least 10 000 g.

After centrifugation, 900µL of the supernatants were transferred to a new tube without disturbing the pellet. To these, 200 µL of chloroform was added and the contents were mixed for 15 seconds with vigorous shaking and incubated at room temperature for 3 min. Then again, the tubes were centrifuged for 15 minutes at 4 °C with at least 10 000 g. Carefully 400 µL from the upper phase was taken into a new 1.5 ml Eppendorf tube and 200 µL of chloroform treatment is repeated. The tubes were shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes, centrifuged for 5 min at room temperature with at least 10 000 g. Carefully 400 µL from the upper phase was taken into a new 1.5 ml Eppendorf tube without contacting the interphase and 1 volume isopropanol was added to each tube. They were all mixed by inverting several times and incubated for 10 minutes at room temperature.

Afterwards, RNA was obtained in the pellet by centrifugation for 10 minutes at room temperature with at least 10 000 g. The supernatants were discarded and pellets were washed with 1 ml of 70% ethanol with 3 minutes incubation at room temperature. After a centrifugation for 5 minutes at room temperature and max speed, supernatants were discarded again. An additional 15 second spin was made by centrifugation to collect the pellet at the bottom of the tube and remove any

visible liquid. The pellets were air dried for 10 minutes in the sterile hood avoiding overdry.

Finally, 40-50 µL DEPC-water was added to each one and the tubes were incubated for 15 minutes at 65ºC to dissolve pellet, and vortex occasionally until it dissolves. RNA can be immediately used after determination of its quantity and quality or kept at -80 C for further use.

2.2.4.4.2. Determination of RNA quantity and quality

The RNA concentration is determined by measuring absorbance at 260 nm on a spectrophotometer in 10 mM Tris/HCl pH 8.0 (one absorbance unit = 40 µg/mL RNA). The A260/A280 ratio should be approximately 2.0, but figures between 1.8 and 2.1 are considered acceptable. The RNA concentration should be greater than 1.1 µg/µl to accept it as appropriate isolation. After measuring the optical densities the concentration of RNA can be calculated as follows:

$$[RNA]\ (\mu g/mL) = 40 \times \text{Dilution Factor} \times OD_{260}$$

The integrity of the RNA is tested by agarose electrophoresis. For this RNase-free 1% agarose gels in TAE are prepared with DEPC - water. If the equipment was used without DEPC-water before, the tank, tray and comb was treated with 3% hydrogen peroxide for 15 minutes. Approximately 2 µg RNA was loaded from each sample in RNase-free DNA loading buffer and run for 30-60 min at 80-100 V. The rRNA bands should be clear without any obvious smearing patterns.

2.2.4.4.3. Northern Blotting

Vacuum-dried 30 µg of RNA samples were dissolved in 20 µL of freshly prepared sample buffer (Appendix I). For denaturation, the eppendorf tubes were

incubated at 65^0C for 10 minutes. The samples were loaded to 1% agarose gel containing, 6 mg/ml ethidium bromide, 1X MOPS (Appendix I) and 6% formaldehyde. Electrophoresis was run with 1X MOPS as buffer and 5 V/cm for 2-3 hours.

A Hybond-N^+ nylon membrane, 3 pieces of 3 MM paper and paper towels (at least 5 cm) having same sizes with the gel were cut. A plastic tray was half filled with transfer buffer (Appendix I) and a platform was established and covered with a wick made from two long sheets of 3 MM paper. The papers were saturated with transfer buffer and the gel was placed on the wick platform. The circumferences of the gel were covered with parafilm to prevent the absorption of transfer buffer by paper towels directly.

A Hybond-N^+ nylon membrane was placed on the gel and wetted with transfer buffer. Seven sheets of 3 MM paper was placed on the membrane one by one and each of them was wetted with transfer buffer after placement. After each step rolling a glass pipette prevented the formation of air bubbles. Absorbent paper towels were placed on 3 MM papers. Finally a glass plate and a 1 kg weight were placed on the towels and the capillary transfer was allowed to proceed overnight. Next day the transfer apparatus was dismantled and the nucleic acids were fixed to the membrane by illuminating the membrane with UV light for 45 seconds.

2.2.4.4.4. Probe Labeling, Hybridization and Detection

After the blotting, Amersham Biosciences' Gene Images AlkPhos Direct Labeling and Detection System with CDP-*Star* was used.

The membrane was placed in hybridization tube and pre-hybridized with the hybridization buffer (which was preheated to 50°C) (Appendix I) for at least 1 hour at 55^0C in a rolling tube hybridization oven. For 1 cm^2 membrane, 0,25 ml hybridization buffer was used.

During pre-hybridization the probe that will be used for hybridization was prepared. Labeling of the probe was done according to Amersham Direct DNA Labeling System. From stock solution, DNA to be the probe was prepared as 10 ng/µL concentration. For 1 ml hybridization buffer, 5-10 ng probe was needed. 10 µL of cross linker solution was diluted with 40 µL of water supplied with kit to obtain the working concentration. 10 µL of diluted DNA was placed into an eppendorf tube and denatured by heating for 5 minutes in a vigorously boiling water bath.

Eppendorf tubes were immediately cooled on ice for 5 minutes and centrifuged to collect the contents at the bottom of the tube. 10 µL of reaction buffer was added to the cooled DNA and the contents were mixed thoroughly but gently. 2 µL of labeling reagent was added to the eppendorf tube and the contents were mixed thoroughly but gently. Finally, 10 µL of cross linker working solution was added and the contents were mixed thoroughly. The labeling reaction can be scaled up by increasing the volume of all components of the labeling reaction pro rata; DNA, reaction buffer, labeling reagent and cross linker working solution. The contents were collected at the bottom of the tube by micro centrifugation. Eppendorf tube was incubated at 37^0C for 30 minutes. The prepared probe can be used immediately or kept on ice for up to 2 hours.

The labeled probe was then added to the pre-hybridized membrane and left for hybridization at 55^0C overnight in rolling tube hybridization oven. The following day, membrane was first washed with primary wash buffer and then secondary wash buffer. The blots were transferred to preheated primary wash buffer and washed two times at 55^0C for 10 minutes with gentle agitation. The blot was transferred to a clean container and washed twice with excess secondary wash buffer working solution at room temperature for 5 minutes. Secondary wash buffer working solution should be used immediately after preparation.

The excess secondary wash buffer working solution was drained from the blot by touching the corner of the blot against a clean surface and the blot was transferred on a glass surface sample side up. 30-40 μL/cm^2 of detection reagent was pipetted on to the blot and the blot was leaved for 2-5 minutes. Excess reagent was drained off the membrane. The blot was wrapped in a stretch film and put into a film cassette in DNA side up position. A sheet of film was placed on the blot in dark room while red lights are on. Finally, the cassette was incubated at room temperature for 3 hours, and then the film was taken and developed.

2.2.5. Plant Hypersensitivity Response Assays

The HR assay was performed according to Huang *et al.* (1995). Fully expanded tobacco leaves were wounded to form tiny holes on the lower surface of the leaves with a 25-gauge needle 100μl of overnight grown bacteria were infiltrated with a 1 ml blunt-end syringe through the hole. The infiltrated plant was incubated at greenhouse conditions and photographed.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Verification of *pflp* within the plasmids

In order to prove that *pflp* gene is present in the plasmids of *A. tumefaciens* cells, plasmid isolation and PCR studies were performed. Plasmid isolation from *Agrobacterium* cells was rather a simple task and a well-set protocol in our laboratory and they were used as positive control for further molecular studies throughout the study whenever needed.

PCR was carried out with *pflp* specific primers as described before and the amplicons were run in 0.8% agarose gel, then the gel was photographed. The PCR result was shown in Figure 3.1.

The arrow on DNA marker in lane 1 shows 500 bp band and lane 2 was blank (without plasmid) which was used as negative control. The remaining lanes, from 3 to 8, were for 3 individual isolations with duplicates as the expected 431 bp fragment

3.2. Determination of Lethal Hygromycin Concentration

The antibiotic hygromycin B is commonly used as a selection agent in gene transfer studies of plants when the hygromycin resistance gene, *hph* is utilized. It is an aminoglycosidic antibiotic and kill cells by inhibiting protein synthesis. The

resistance gene codes for a kinase (Hygromycin phosphotransferase, HPT) that inactivates Hygromycin B through phosphorylation.

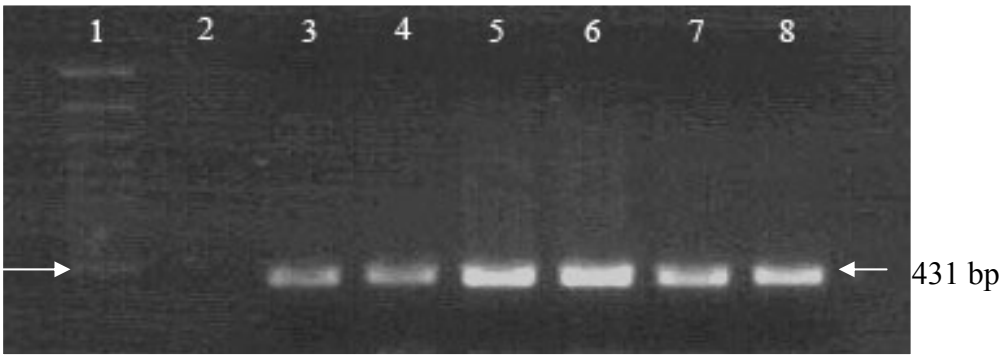

Figure 3.1 PCR result of *pflp* amplification of plasmids after isolation.
Lane 1. Gene Ruler® DNA Marker, Lane 2. Blank, Lanes 3-8 pSPFLP

The working concentration for the purpose of selection varies with cell type, media, growth conditions and cell metabolic rate. Commonly used concentrations for selection are 200 mg/L for mammalian cells, 20-200 mg/L for plant cells & bacteria cells and 200-1000 mg/L for fungi (AG Scientific, 2005). In a study with orchids, 5 mg/mL had been used for selection by Liau *et al* (2003). Since pSPFLP contained *hph* in T-DNA region, in order to use it in selection, the optimum concentration for this study should be tested experimentally

For this purpose, leaf discs were prepared and placed on M9274 medium containing 0,1 mg/L NAA, 1 mg/L BA and varying concentrations of hygromycin which were 5 mg/L , 10 mg/L , 25mg/L , 50 mg/L and 100 mg/L . It is observed that 25 mg/L hygromycin concentration would be enough to kill wild-type and non-transformant plants (Figure 3.2) , so that this concentration is used both for selection and for negative controls during the study.

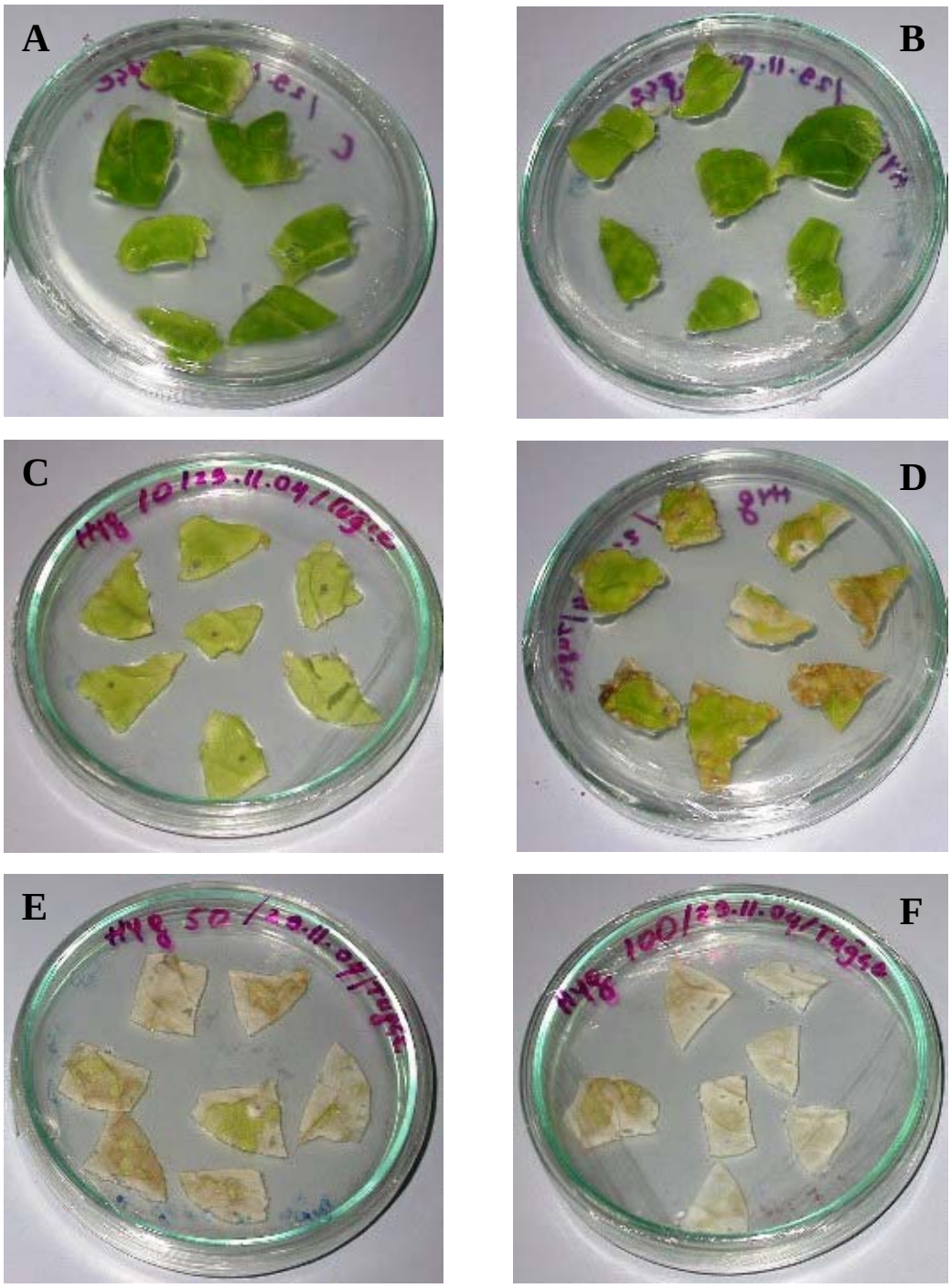

Figure 3.2 Results of Lethal Hygromycin Concentration Determination. A=control, no hygromycin B= 5 mg/L C= 10 mg/L D= 25 mg/L E= 50 mg/L F= 100 mg/L

3.3. Generation of Transgenic Tobacco Lines

For tobacco, there are various transformation protocols, but *Agrobacterium* mediated protocol was preferred (Öktem *et al*., 1994) since it has been a well established and highly efficient procedure in our laboratory.

3.3.1. Callus Induction

The tobacco leaves which leaf discs were prepared from, were almost at the same age and had been grown under the same conditions. Following the initial incubation of all the explants with Agrobacterium cells, the leaf discs together with untreated ones, were kept on non-selective media for two days which was called as cocultivation period. Then, all the explants including the non-transformed plants controls were washed thoroughly in MS medium containing the antibiotic cefotaxime (750 mg/L) in order to supress the remaining bacterial growth.

For every individual transformation experiment, there were also positive and negative controls. Positive control means non-transformed leaf discs, they were not treated with *Agrobacterium* cells and they were placed on nonselective MSA media. Callus formation was seen in positive control leaf discs and this indicated that our regeneration system was appropriate. On the other hand, negative control means non-transformed leaf discs, again they were not treated with *Agrobacterium* cells but they were placed on selective MSB media. Negative control leaf discs were not able to form calli and necrosis was observed due to toxic hygromycin, this showed our selection system was appropriate. Transformed leaf discs were also placed on selective MSB media. After 3-4 weeks, formations of calli were started as a result of *hph* gene transformation which inactivates the toxic hygromycin in the media.

Figure 3.3 shows the formation of shoots on calli of transformed leaf discs after 6 weeks. Positive controls showed callus and shoots formed much faster than the transformed ones, even *hph* prevents the toxic effects of hygromycin in transformed cells, the growth is retarded. The transformation experiments were repeated 3 times in order to confirm the reliability of the results.

Figure 3.3 A photograph showing positive and negative control and *Agrobacterium* treated explants after 6 weeks.

In addition to this, it was observed that more than 60% percent of *Agrobacterium*-treated leaf discs were survived on selective medium containing 25 mg/L hygromycin and these gave the first line of evidence that the gene of

interest has been transferred to the plant. Under same conditions most of the untreated explants were died and support the success of transformation.

3.3.2. Shoot and Root Development

After 5-6 weeks from transformation, shoots started to emerge from the calli and they became ready to be handled in the following 3-4 weeks. The shoots were cut at the base and transferred to MSC Medium for root induction. Figure 3.4 shows the development of a putative transgenic shoot in MSC Medium with both negative and positive controls. Hygromycin concentration of the medium has not been changed and the ability of root formation in transformed shoots can be considered as another line of evidence that *hph* is present within the cells. Root formation has been observed in most of the jars within 2 weeks. After several sets of transformation experiments, the number of transgenic lines has reached 23.

3.3.3. Development in Soil

An essential part of the regeneration cycle is development of the plantlets in soil. It is necessary to obtain successive generations in order to assess the stability of the gene of interest within the plant genome. In order to accomplish this task, nearly one month later, the regenerated plantlets have been transferred from jars to soil. At this point, the number of lines was decreased to 15 depending on their growth and health.

The plantlets in jars were very sensitive and fragile. Thus, before taken to the greenhouse, the plantlets in pots were kept in an environmental growth chamber at 25°C with 70% humidity at a photoperiod of 16 hours for 1 month. In order to acclimatize the plantlets, the pots were covered with stretch film to avoid excessive evaporation and drying of the plantlets first 5 days

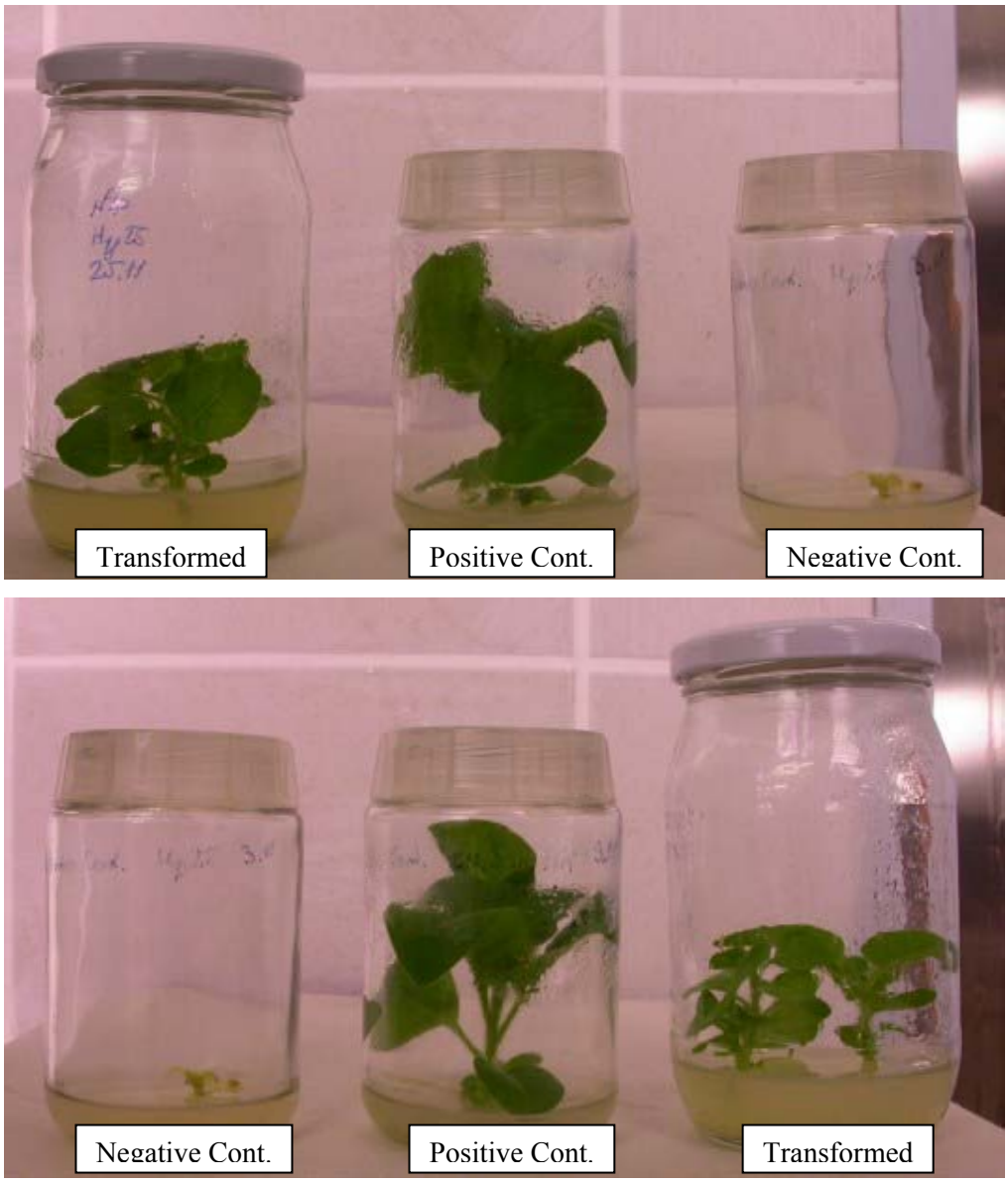

Figure 3.4 Root formation in putative transgenic plantlets in MSC medium. Negative control which was a shoot from positive control grown calli, could not develop root and died in hygromycin containing medium, whereas positive controls developed roots rapidly in medium without hygromycin.

Figures 3.5, 3.6 and 3.7 demonstrate the development of different putative transgenic lines in growth chamber and in greenhouse, respectively. The plants taken to soil have started to flower in 2-2.5 months' time depending on the weather conditions and they were self-pollinated. In order to prevent cross-pollination between the plants, the flowers were covered with plastic bags during flowering time. The T_1 seeds were ready to be collected in 3-3.5 months. All of the plants taken to soil were fertile and it was also observed that the fully grown plants did not show any morphological difference compared with the wild type plants in soil.

Figure 3.5 The growth of putative transgenic line 6 in soil. The photograph is taken 10 days after transferring to soil.

Figure 3.6 The growth of putative transgenic plants in growth chamber. The photograph demonstrates one group of independent lines 4 weeks after transferring to soil.

Figure 3.7 The growth of putative transgenic plants in greenhouse conditions. The photograph demonstrates one group of independent lines 9 weeks after transferring into soil.

3.4. Analysis of T_1 progeny

From 15 independent transgenic lines which gave seeds in greenhouse, 5 lines were randomly selected within the best grown and healthiest ones and the following analysis were applied to these 5 lines: T1, T3, T6, T9 and T10. Besides analyzing the Mendelian inheritance pattern of T_1 seeds and GUS histochemical assays, molecular studies at DNA and RNA level were carried out.

3.4.1. Mendelian Inheritance Pattern

With the intention of determining the segregation pattern of transferred gene in T_1 transgenic tobacco plants, the seeds from randomly selected 5 lines were germinated in selective media containing 25 mg/L hygromycin. For each line approximately 100 seeds were used and the numbers of survivors in selective media were counted. The results are given in Table 3.1. The expected ratio for Mendelian inheritance is 3:1 and the data was analyzed by Chi-Square Analysis with $p\ (\chi^2_1 \leq 2{,}706) = 0.9$. The appearances of transgenic seed in selective media are shown in Figure 3.8. As can be seen from the photographs, T6 seeds grew faster than the other lines in selective medium.

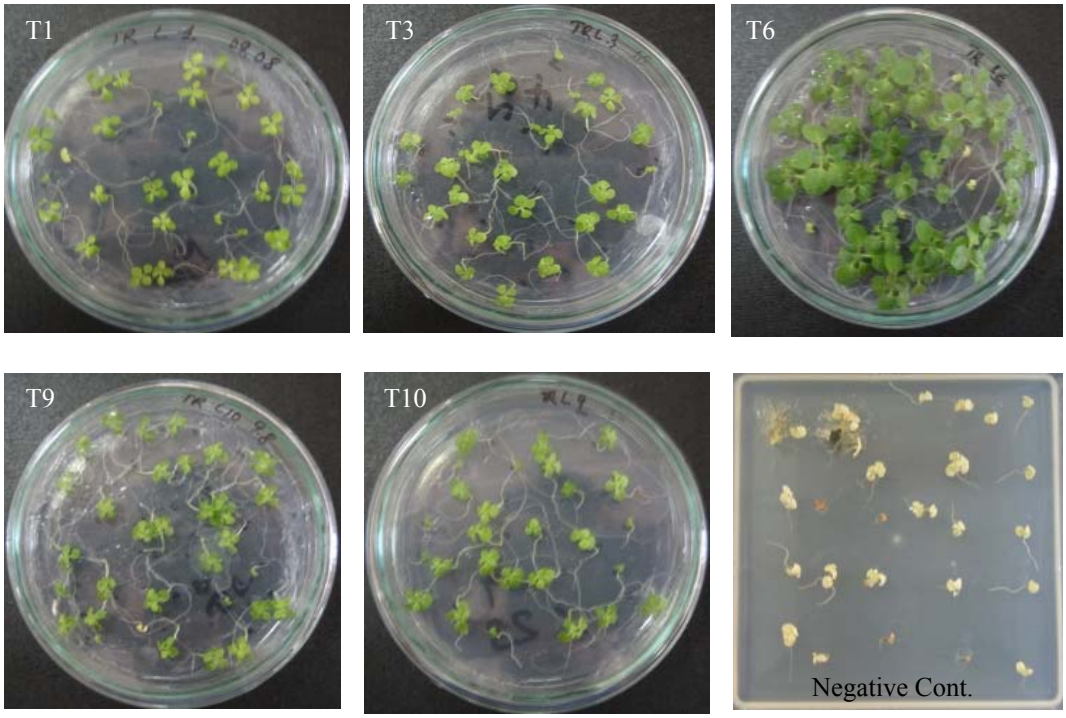

Figure 3.8 The growth of T_1 seeds in selective media. Wild type seeds used as negative control in selective medium. The photographs were taken after 6 weeks.

Table 3.1 Mendelian Inheriatance Analysis of T_1 progeny, $p(\chi^2_{0.90,1}) \leq 2{,}706$

Lines	Total # of Seeds	# of Germinated Seeds	χ^2	Decision
T1	99	68	2,64983165	Fail to Reject
T3	97	76	0,580756014	Fail to Reject
T6	98	77	0,666666667	Fail to Reject
T9	98	74	0,013605442	Fail to Reject
T10	99	71	0,569023569	Fail to Reject

3.4.2. GUS Histochemical Assay

T-DNA region of pPFLP contains *gus A* gene which enables the transgenic tissues to form blue color following their incubation in X-gluc chromogenic substrate. So, in order to monitor the gene expression in T_1 leaves histochemical

GUS staining was performed. Besides T3 line, other randomly selected lines gave GUS positive results (Figure 3.9)

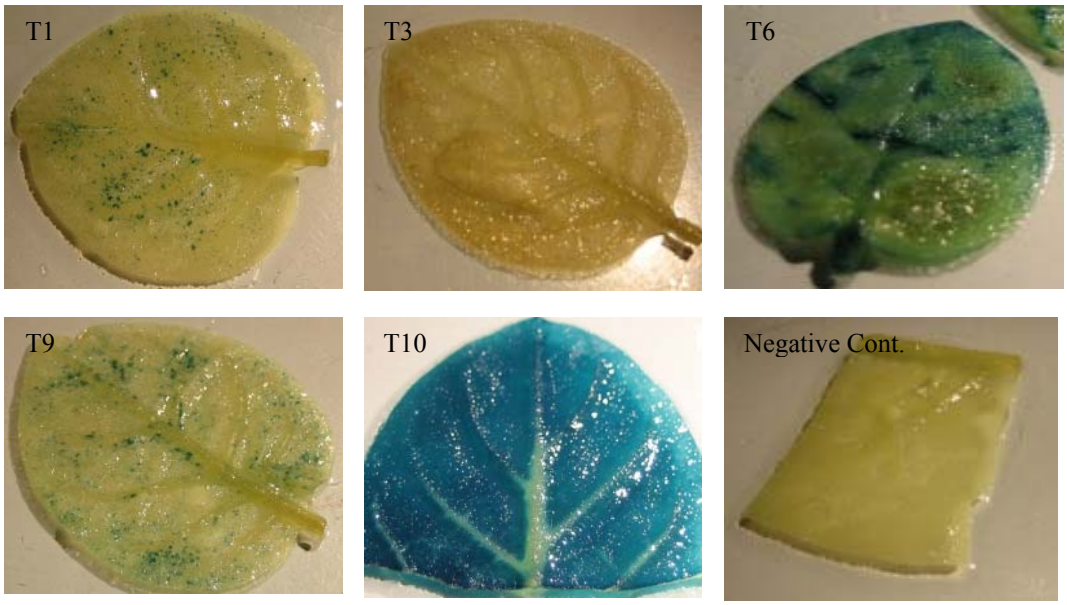

Figure 3.9 Expression of GUS in leaves of transgenic lines. Negative control is wild type tobacco.

GUS expressions in T10 and T6 were intensive; in fact the whole leaf explant was colored for T10. At a lesser amount, T1 and T9 expressed GUS with local colorings whereas T3 did not show any coloring pattern even under microscopic observation meaning that GUS expression is absent.

3.4.3. Molecular Analysis of T1 via PCR

3.4.3.1. PCR with *pflp* and *hph* specific primers

PCR analysis was used to obtain an idea about the verification of gene transfer to the T_1 transgenic lines. For this purpose from each putative transgenic

line CTAB DNA extraction was carried out. PCR analysis was done with *pflp* and *hph* primers. For the negative control, the genomic DNA of wild type plant was isolated and used in PCR analysis where as, for the positive control, pure plasmid pPFLP was isolated and used. Also a blank control is also used during PCR which contains all the elements of a typical PCR but not any DNA sample. Reactions are carried out twice to obtain reliable results. The PCR results with *pflp* and *hph* specific primers are shown in Figure 3.10.

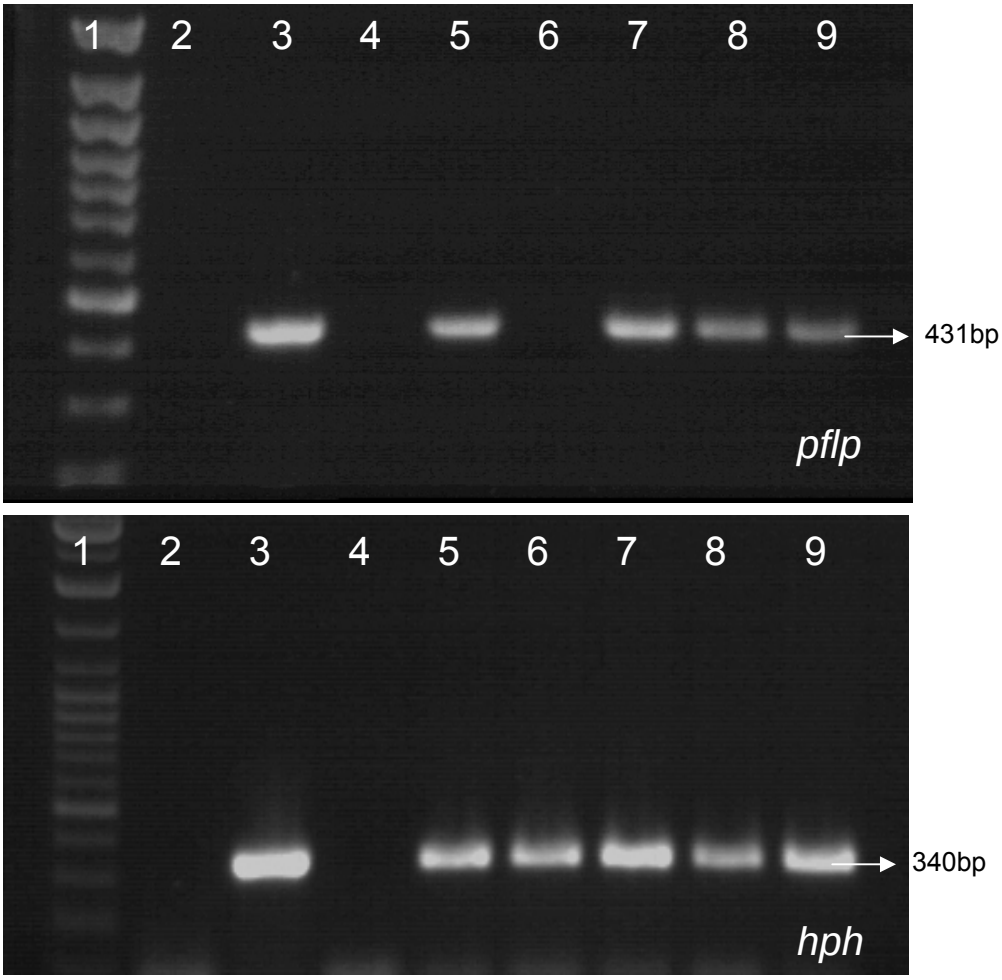

Figure 3.10 The agarose gel (0.8 %) photographs of PCR analysis for *pflp* and *hph* gene. DNA marker was Gene Ruler 100bp Plus from Fermentas. The samples used in PCR:

Lane 1. DNA marker **Lane 2.** Blank control **Lane 3.** pPFLP **Lane 4.** Wild-type **Lane 5.** T1 **Lane 6.** T3 **Lane 7.** T6 **Lane 8.** T9 **Lane 9.** T10

It can be seen that in selected transgenic lines, 431 bp for *pflp* and 340 bp for *hph* fragments were amplified as expected and PCR results reveal that *pflp* and *hph* genes have been successfully integrated and inherited into the genome of selected T_1 lines except T3. This line gave negative result for *pflp* gene specific PCR, where as it gave positive result for *hph* gene. This result is interesting because when T3 seeds had been grown on selective medium, they could withstand the toxic effects of hygromycin and survived, however T3 also gave negative GUS reaction. Considering all these, it can be assumed that T3 contains the part of T-DNA which *hph* is present but does not contain the remaining *pflp* and GUS-GFP fusion (Appendix A).

3.4.3.2. Sequence Analysis of PCR products

For the purpose of ensuring the amplicons, sequence analysis was carried out. Only line 6, which gave positive results for GUS histochemical staining and positive reactions for both PCRs with *pflp* and *hph* primers, was selected for this analysis.

The analysis was done with the products of PCRs which were carried out with *pflp* gene, CaMV 35S promoter and NOS terminator specific primers, then the products were given to Sequence Analysis Unit, Central Lab., METU with amounts requested by the unit. According to the unit, a second PCR, sequencing PCR was performed with either forward or reverse primers for each three gene, so there were two results obtained for each gene. The results were examined with Chromas v2.23 software program. With the "BLAST search" option that was built in the software, sequences were compared with previously characterized genes from the internet data base of NCBI and the results for each gene and primer were given in Table 3.2.

Table 3.2 Sequence Analysis Results of PCR products given as sequence identity compared with previously characterized genes.

	Forward	Reverse
CaMV 35S P	96%	99%
NOS T	97%	95%
pflp	99%	99%

The results show very high identity when comparison is made with the genes that are previously characterized and available in the internet. Sequence analysis of CaMV 35S promoter and NOS terminator gene results were found in pCAMBIA 1304 and pCAMBIA 1305.2 complete sequences, respectively, which our plasmid, pPFLP was derived from, where as *pflp* gene results were found in *Capsicum annuum* ferredoxin-like protein mRNA, complete sequence, which is the sequence that PFLP was first characterized. From the results, the averages of the identity values were calculated from three values for CaMV 35S promoter and two for NOS terminator genes, and this is consistent since the T-DNA region contains three and two copies of CaMV 35S promoter and NOS terminator genes, respectively. The reference number and values, scores, expects and query-subject matching for each analysis was given in Appendix H.

3.4.3.3. Real Time-PCR Analysis

Another performed analysis was Real Time (RT) - PCR which was carried out by the service of RT-PCR Unit of Central Lab., METU. According to the unit, Roche Applied Science's High Pure GMO Sample Preparation and LightCycler ® GMO Screening Kits were used. The former kit is specially designed for the isolation of DNA from raw material and food products of plant origin for PCR analysis, where as the latter one is a PCR kit for the qualitative detection of genetically modified plants using the LightCycler ® System.

The GMO Screening Kit provides a molecular method for detection of the most commonly used control sequences of genetically modified plants. These sequences are the 35S promoter of CaMV and the 3' -untranslated region (terminator) of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. 35S and NOS sequences are found in more than 95% of the presently available GMO crops (Roche Applied Science, 2005). So the working principle of the kit depends on simultaneous amplification and detection of specific DNA sequences using 35S- and NOS-specific primers and Hybridization Probes. Detection of either sequence indicates a high probability of GMO presence in the investigated sample material.

So in order to confirm 35S promoter and NOS terminator within transgenic plants RT-PCR is carried out. Two individual PCR was carried out in the Lightcycler namely as target and reference reactions. The target reaction was performed with 35S- and NOS-specific primers in order to detect any transgene presence, whereas the reference reaction only detects whether the sample contains plant DNA or not, so act as an internal control. From Figure 3.11, it is understood that all wild type and transgenic lines DNA gave nearly same positive results, considering that reference values just represent the presence of plant genomic DNA, it is consistent since equal amounts of DNA was used in the reactions. However, comparing with the positive control that the kit contains, our samples started to fluoresce much earlier, only at 12^{th} cycle, but positive control at 29^{th} cycle, and this phenomenon can be explained by the fact that our samples contained higher concentrations of DNA than the kit's reference sample.

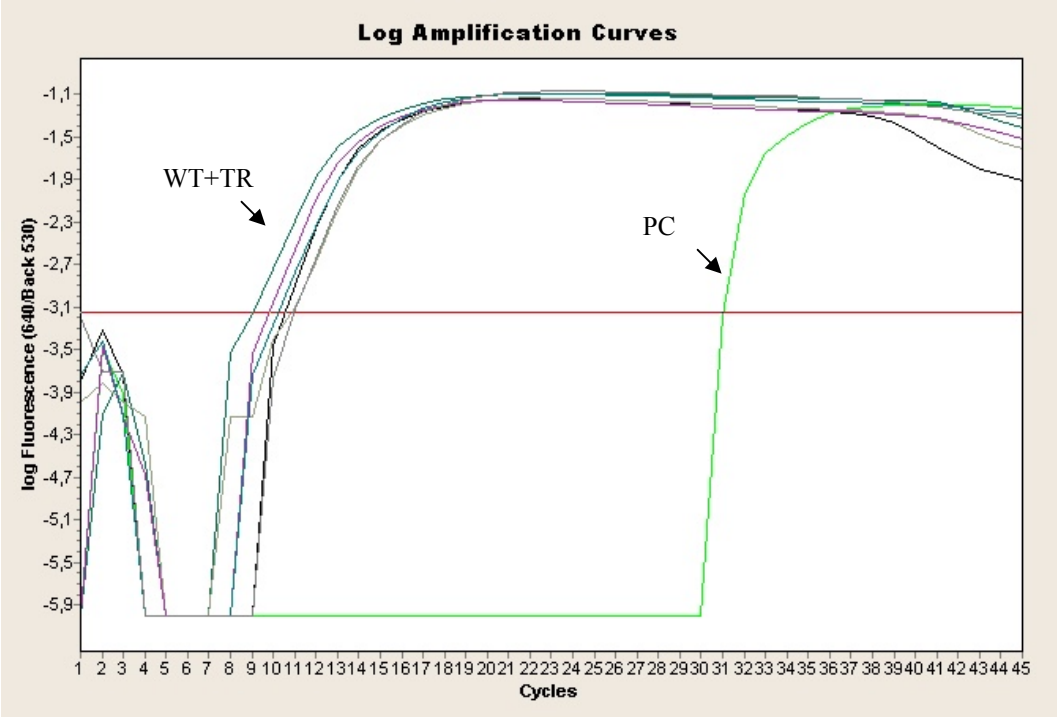

Figure 3.11 Log amplification curves for reference values. The Fluorescence vs PCR cycle number graph shows wild type (WT), transgenic lines (TR) and positive control (PC). The straight line shows the noise band value.

The results of CaMV 35S promoter and NOS terminator RT-PCR analysis were obtained separately and given in Figure 3.12 and 3.13 respectively. They both indicate the presence of 35S and NOS transgenes within the transgenic lines.. The data for target reactions show that all the transgenic lines started to fluoresce at the same cycle number, around 21st and 22nd cycle, whereas the positive control again started its peak at 31st cycle. This difference can be explained as above, due to the differences in DNA concentration. However, the sample from wild type which was the negative control of the analysis, also gave a positive value. A typical negative control should give no fluorescence but in our analysis, it started to fluoresce at 35th cycle even though the fluorescence started very late in the reaction process.

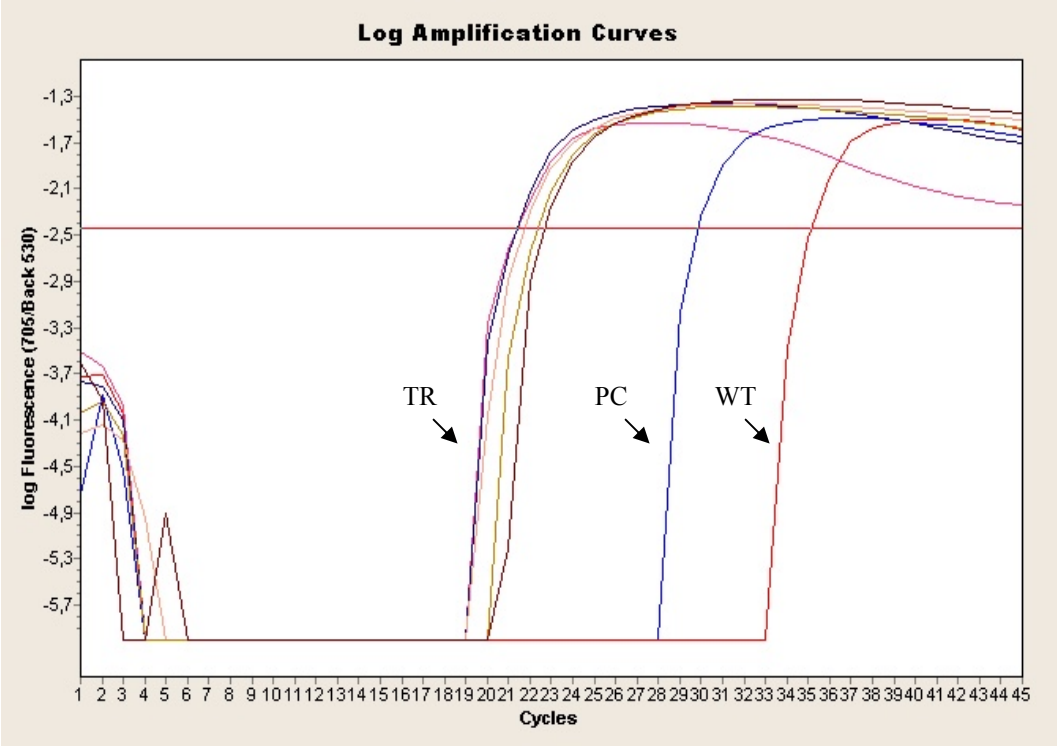

Figure 3.12 Log amplification curves for CaMV 35S promoter target values. The Fluorescence vs PCR cycle number graph shows wild type (WT), transgenic lines (TR) and positive control (PC). The straight line shows the noise band value.

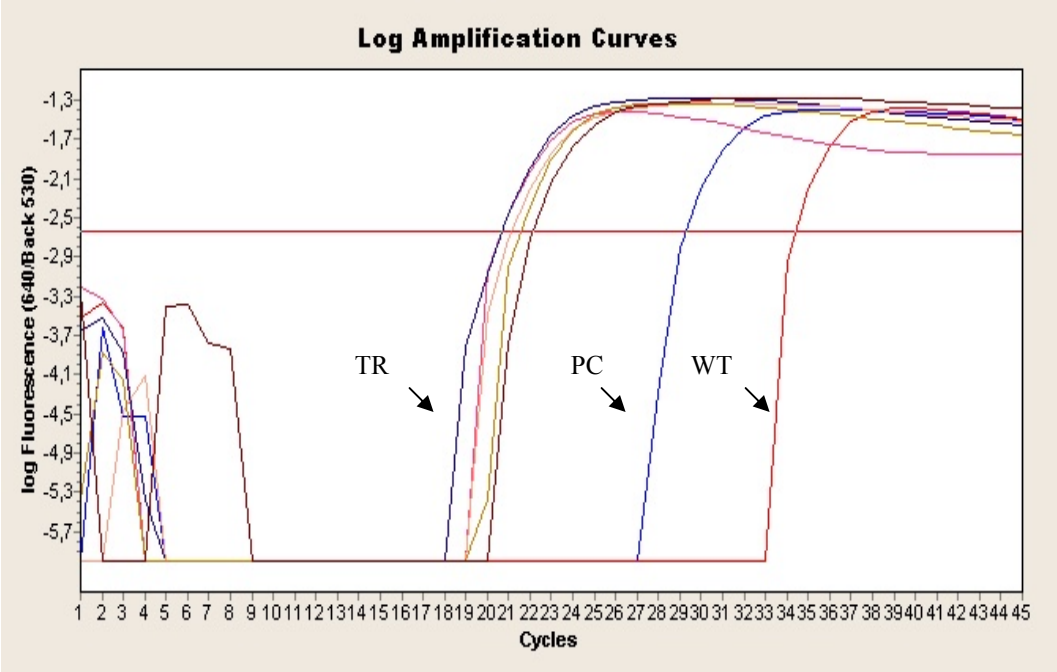

Figure 3.13 Log amplification curves for NOS terminator target values. The Fluorescence vs PCR cycle number graph shows wild type (WT), transgenic lines (TR) and positive control (PC). The straight line shows the noise band value.

In order to be sure, the analysis was repeated, but this time only wild type sample was used. In Figure 3.14 and 3.15, results for 35S and NOS values were given respectively, together with the reference values. Both results showed that the fluorescence of wild type remained below the noise band line which is determined by the software automatically, and it was considered that no transgene was present in wild type plants.

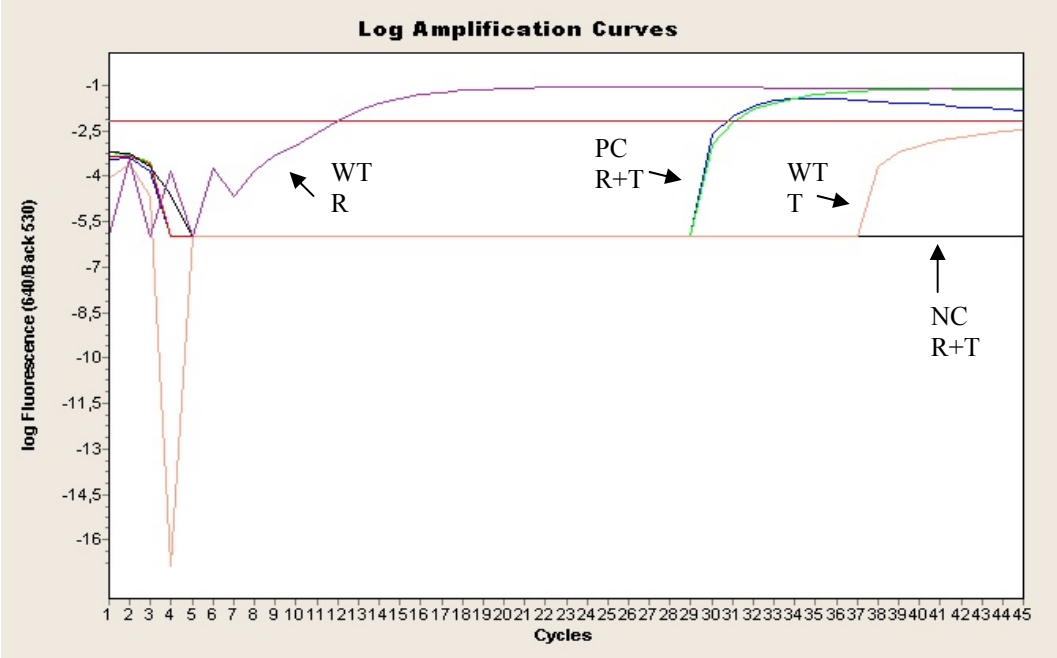

Figure 3.14 Log amplification curve for reference (R) and 35S target (T) values. The Fluorescence vs PCR cycle number graph shows wild type (WT), transgenic lines (TR), positive control (PC) and negative control (NC). The straight line shows the noise band value.

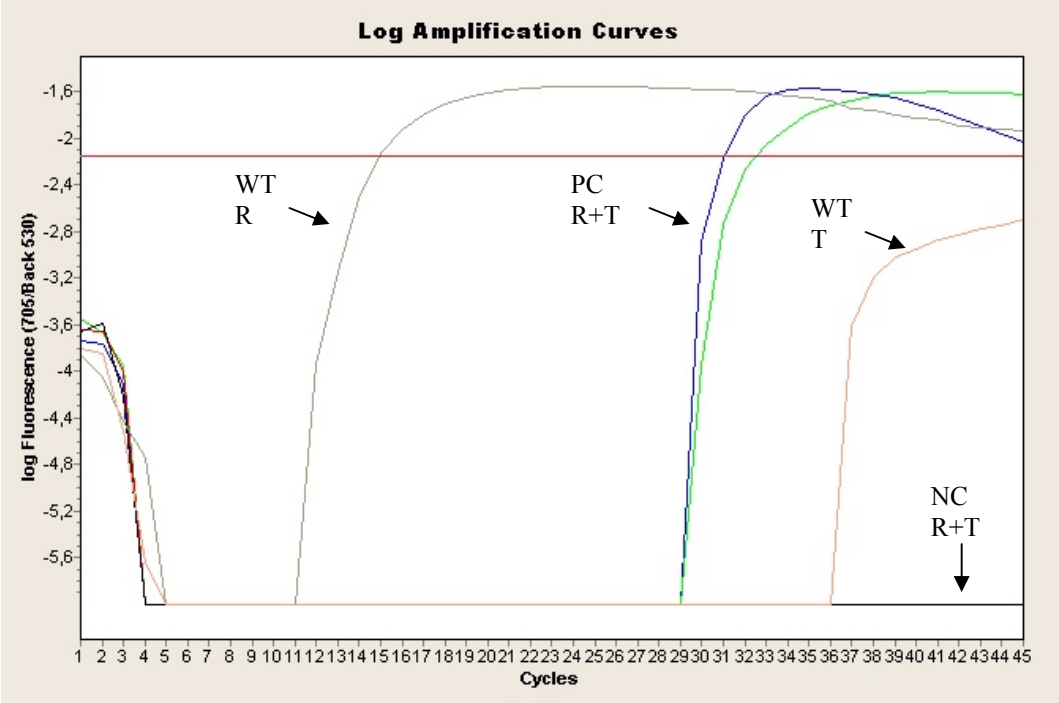

Figure 3.15 Log amplification curve for reference and NOS target values. The Fluorescence vs PCR cycle number graph shows wild type (WT), transgenic lines (TR), positive control (PC) and negative control (NC). The straight line shows the noise band value.

Ultimately, RT-PCR analysis results can be summarized as in Table 3.3 below.

Table 3.3 An overall look to the RT-PCR analysis results

Sample	Plant DNA	CaMV 35S promoter	NOS terminator
T1	+	+	+
T3	+	+	+
T6	+	+	+
T9	+	+	+
T10	+	+	+
Wild type	+	-	-

3.4.4. Expression Analysis of *pflp* in T_1 Transgenic Plants

In order to evaluate the transgene expression of *pflp* in transgenic lines, Northern Blot analysis was also performed. For this purpose, total RNAs were isolated from wild-type plant which is used as control and from each selected transgenic line. According to the spectrophotometric analysis, the volume for 2 µg of total RNA from each line and wild-type was calculated and electrophoresed (Figure 3.16). rRNA patterns for all samples were clear without any obvious smearing patterns and equal loading was verified with ethidium bromide staining as an internal control for blotting.

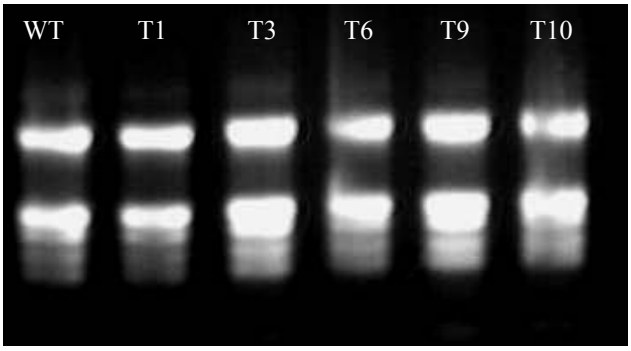

Figure 3.16 Total RNA isolated from wild-type and the selected transgenic lines.

For blotting, 20 µg of total RNA from all samples were electrophoresed again in 1% denaturating agarose gel and then they were transferred to a nylon membrane. After pre-hybridization, hybridization and stringency washes, detection reagent was applied to membrane and signals were detected. Expressions of *pflp* gene in transgenics are shown in Figure 3.17.

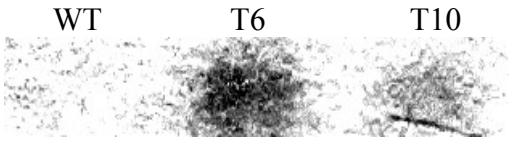

Figure 3.17 Northern Blot analysis of wild-type and transgenic lines T6 and T10.

It was observed that the levels of pflp mRNA transcripts were not highly expressed in all selected lines. Just T6 and T10 transgenic lines showed high expression, where as for wild-type, as expected, T1, T3 and T9, not as expected, showed no observable level of *pflp* expression. In fact equal amounts of total RNA was used for each line, but the main reason could be about stringency and hybridization conditions during blotting which might not be applied in optimum. Nevertheless, signals were detected for T6 and T10 samples and in a way this shows their expression was higher than other lines. Liau *et al.* (2003) also found similar results in transgenic orchids obtained using the same plasmid.

3.4.5. Overall Evaluation of T_1 Transgenic lines

In addition to previously discussed GUS histochemical and molecular analysis, which showed the presence of *uid A, pflp* and *hph* genes, Northern Blot analysis showed that *pflp* was highly expressed in lines T6 and T10. However, lines T1 and T9 showed the presence of *pflp* and *hph* genes by PCR analysis, but their GUS histochemical coloration was not intense as T6 and T10. This result is not unexpected since the T-DNA integration occurs randomly and in our case,for lines 6 and 10, the T-DNA must be inserted to an actively expressed site in the plant genome. On the other hand, T3 just gave positive PCR result for *hph* gene, but not for *pflp*, and no color formation was observed when GUS histochemical assay was performed, so this line can be categorized as escape. All these data was summed up in Table 3.4.

Table 3.4 The summary of GUS and molecular analysis results. (+++) in GUS staining indicates the intensive coloring. WT= Wild-type

Line #	PCR of *pflp*	PCR of *hph*	GUS staining	Northern B.
1	+	+	+	-
3	-	+	-	-
6	+	+	+++	+
9	+	+	+	-
10	+	+	+++	+
WT	-	-	-	-

3.5. Plant Hypersensitivity Response Assays

In this part of the study, the aim was to observe the increase in harpin-mediated HR with the interaction of PFLP. For this, plant material was the putative T_0 transgenic lines 1 and 6, they were chosen when their seeds, T_1 transgenics were randomly selected and before any molecular analyses were performed. Also for inoculations, two different pathovars of *Pseudomonas*

syringae species which produce harpin were selected: *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *pisi* . In this preliminary test, HR was observed in transgenic lines after 6 days with bacterial injection (Figure 3.18). Putative transgenic lines 1 and 6 showed HR with a thin, film like appearance around the infection area. These findings are consistent with other studies performed in rice (Tang *et al.,* 2001), orchid (Liau *et al.,* 2003) and tobacco (Huang *et al.,* 2004) where they obtained HR with harpin producing bacteria.

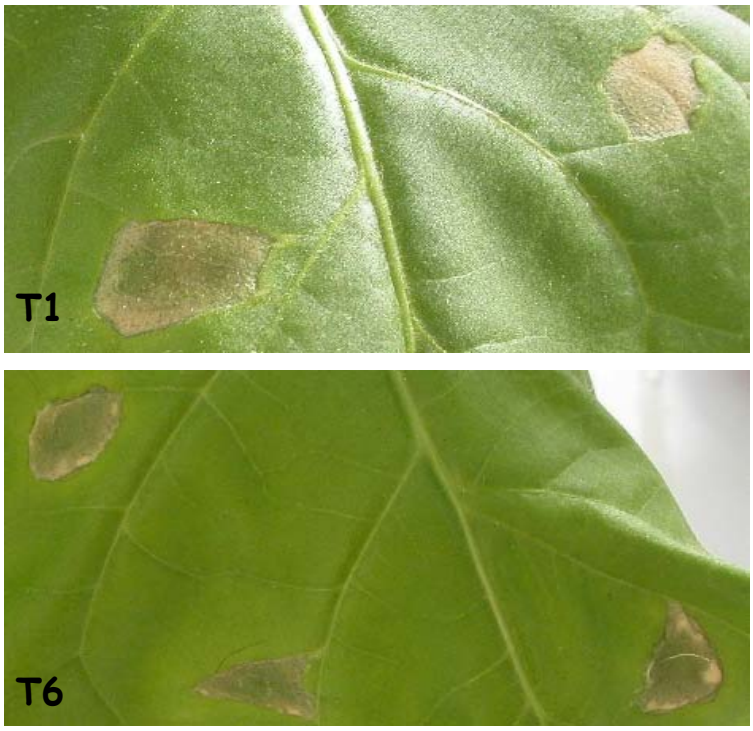

Figure 3.18 Hypersensitive Response given to *P. syringae* by the transgenic lines 1 and 6.

CHAPTER IV

CONCLUSION

The objective of the present study was to evaluate the influence of *pflp* gene expression in transgenic tobacco (*Nicotiana tabacum* cv. Samsun) plants and develop PFLP mediated Hypersensitive Response for various harpin producing pathogens.

To complete this aim, first of all, *pflp* gene was transferred to tobacco via *Agrobacterium* –mediated genetic transformation. The transformed tobacco explants were regenerated in selective media containing hygromycin (25 mg/L). The 23 independent putative transgenic lines were obtained after successive sets of transformation experiments. From these 23 lines, 5 lines were randomly selected and their seeds were grown. These lines were regenerated and transplanted to soil to obtain T_1 generation.

In order to confirm the *pflp* gene presence in the transgenics, several tests were conducted. Mendelian inheritance assay showed that in all five lines the gene was segregated according to Mendelian 3:1 ratio. With GUS histochemical assay, lines 6 and 10 showed intensive coloration where as lines 1 and 9 result in smaller color formation. However, line 3 had resulted in negative GUS reaction.

In addition, PCR amplifications were carried out considering the molecular analyses. PCR reactions with *pflp* specific primers showed positive reaction for all five lines except line 3, whereas with *hph* specific primers showed positive reaction for all lines. Sequencing analyses of PCR products of *pflp*, CaMV 35S

promoter and NOS terminator amplifications showed high identity when comparison is made with the genes that are previously characterized.

Northern Blot analysis was also performed in order to prove the *pflp* mRNA was present in transgenic lines. Only lines 6 and 10, which showed intensive coloration in GUS histochemical assay, gave positive results for Northern Blot analysis. So in conclusion, the analyses altogether confirm that tobacco plant (*N. tabacum* cv. Samsun) has been successfully transformed with *pflp*.

Finally, bioassays have been performed with putative T_0 transgenic lines. HR response against *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *pisi* was observed in the preliminary tests.

Future work would include further hypersensitive response assays with T_1 and T_2 with different pathogenic bacterial species in search for disease resistances in tobacco. In addition, Northern Blot Hybridization of other transgenic plants would be performed to find high-expression transgenic lines and Southern Blot Hybridizations should be carried out to confirm the gene integration into the plant genome.

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APPENDIX A: Plasmid Map

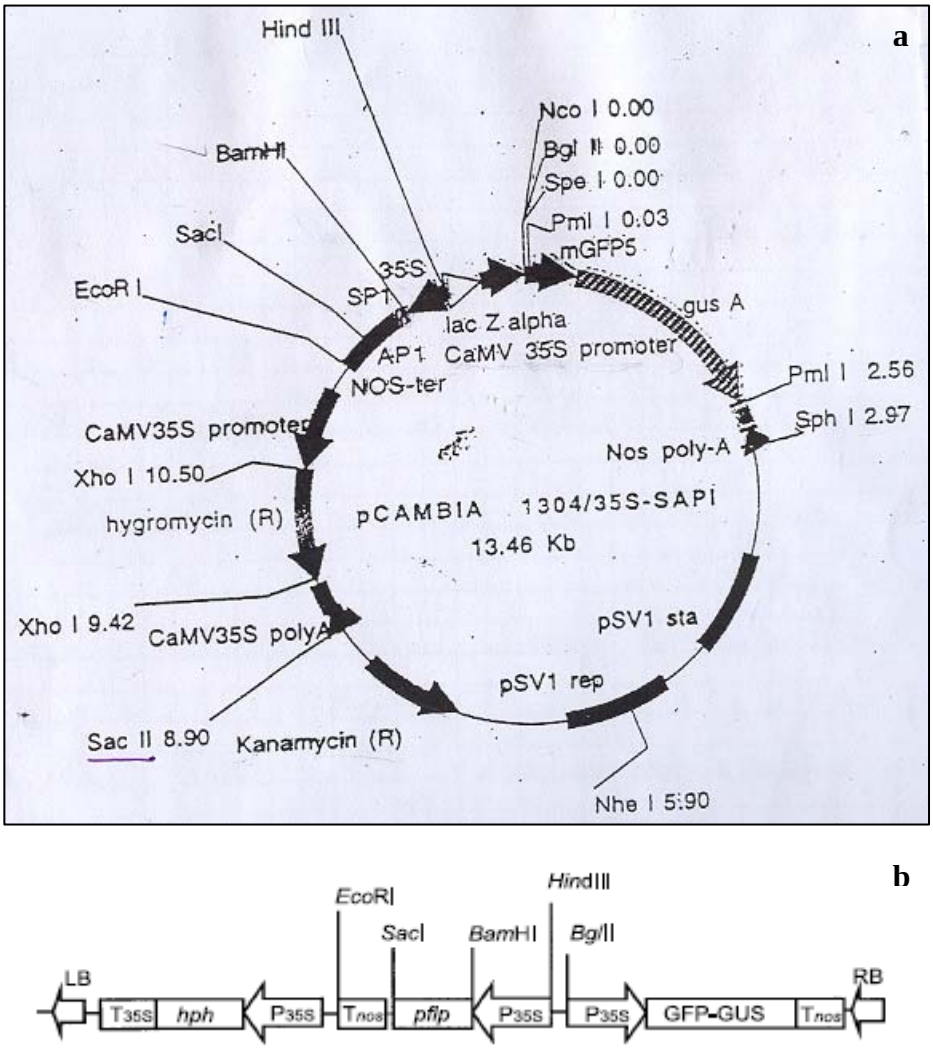

Figure A.1 (a) Complete map of pCAMBIA 1304/35S-SAPI **(b)** T-DNA region of pSPFLP. *Pflp*: sweet pepper ferredoxin-like protein cDNA, *hph* : hygromycin phosphotransferase cDNA sequence; GFP-GUS: green fluorescent protein and β-glucuronidase gene fusion; P35S: cauliflower mosaic virus 35S promoter; *nos*: nopaline synthase terminator sequence; T35S: cauliflower mosaic virus35S terminator sequence; LB: left border; RB: right border

APPENDIX B: Bacterial Culture Medium

YEB Medium

For 1 liter,

Nutrient Broth	13.5 gr
Yeast Extract	1 gr
$MgSO_4.7H_2O$	2 mM
Agar	15 gr

pH: 7.2

LB

For 1 liter,

Yeast Extract	5 gr
Tryptone	10 gr
NaCl	10 gr
1N NaOH	1 ml
Agar	15 gr

pH: 7.4

APPENDIX C: Plant Tissue Culture Media

Table C.1 Compositions of Plant Tissue Culture Media

	M0404	**M5519**	**M9274**
Component	**mg/L**	**mg/L**	**mg/L**
Ammonium Nitrate	1650	1650	1650
Boric Acid	6.2	6.2	6.2
Calcium Chloride anhydrous	332.2	332.2	332.2
Cobalt Chloride.$6H_2O$	0.025	0.025	0.025
Cupric Sulfate.5 H_2O	0.025	0.025	0.025
Na_2EDTA	37.26	37.26	37.26
Ferrous Sulfate.$7H_2O$	27.8	27.8	27.8
Magnesium Sulfate	180.7	180.7	180.7
Magnesium Sulfate.H_2O	16.9	16.9	16.9
Molybdic Acid.$2H_2O$	0.25	0.25	0.25
Potassium Iodide	0.83	0.83	0.83
Potassium Nitrate	1900	1900	1900
Potassium Phosphate monobasic	170	170	170
Zinc Sulfate.$7H_2O$	8.6	8.6	8.6
Organics			
Agar	-	-	8000
Glycine	-	2	2
Myoinositol	100	100	100
Nicotinic Acid	1	0.5	0.5
Pyrodoxine.HCl	1	0.5	0.5
Sucrose	-	-	30000
Thiamine.HCl	10	0.1	0.1
Grams of powder for 1 liter	**4.4**	**4.4**	**42.4**

MSA

For 1 liter,
42.4 gr M9274
1 mg/L BA (from 1mg/L stock solution)
0.1 mg/L NAA (from 1mg/L stock solution)
pH: 5.7

MSB

MSA supplemented with appropriate concentration of selective agent (3 or 5 mg/L of hygromycin from 10 mg/L stock solution).

MSC

42.4 gr M9274 supplemented with appropriate concentration of selective agent (3 or 5 mg/L of PPT from 10 mg/L stock solution).

Liquid MS

For 1 liter,
4.4 gr/L M5519
3% sucrose
pH: 5.5

APPENDIX D: Plasmid Isolation Solutions and TE Buffer

STE

NaCl 0.1 M

Tris.Cl (pH: 8.0) 10 mM

EDTA (pH: 8.0) 1 mM

Sol I

50 mM Glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Sol II

0.2 N NaOH (freshly diluted from 10N stock)

1% SDS

Sol III

5 M Potassium Acetate	60 ml
Glacial Acetic Acid	11.5 ml
dH_2O	28.5 ml

1X TE (Tris-EDTA) Buffer (1 L)

Tris.HCl 10 mM

EDTA 1 mM

All the components are mixed and the volume is adjusted to 1 L with dH_2O.

APPENDIX E: Primers, TAE and TBE buffers

Primers used during PCR amplifications:

pflp

AgSP5 (sense) : 5' – CGG GAT CCC GAT GGC TAG TGT CTC AGC TAC CA – 3'

T_m = 68ºC

Ag3 (antisense) : 5' – CGA GCT CGT TAG CCC ACG AGT TCT GCT TCT – 3'

T_m = 66 ºC

hph

Forward ; 5' – GCT TTC AGC TTC GAT GTA GGA G – 3' T_m = 66 ºC

Reverse; 5' – CAC GCC ATG TAG TGT ATT GAC C – 3' T_m = 66 ºC

10X TBE (Tris Borate) Buffer (1L)

Tris-base 108 g

Boric acid 55 g

0.5M EDTA (pH: 8.0) 40 mL

All the components are mixed and the volume is completed to 1 L with dH_2O.

50X TAE (Tris-acetate) Buffer (1 L)

Tris-base 242 g

Glacial acetic acid 57.1 mL

0.5 M EDTA (pH: 8.0) 100 mL

All the components are mixed and the volume is adjusted to 1 L with dH_2O.

APPENDIX F: GUS Substrate and Fixative Solutions

GUS Substrate Solution

0.1 M $NaPO_4$ Buffer, pH 7.0
10 mM EDTA
0.5mM K-ferricyanide, pH 7.0
0.5 mM K-ferrocyanide, pH 7.0
1 mM X-Glucoronide (dissolved in dimethyl formamide)
10% v/v Triton X-100

GUS Fixative Solution

10% v/v Formaldehyde
20% v/v Ethanol
5% v/v Acetic Acid

APPENDIX G: CTAB Buffer

2X CTAB Extraction Solution (100 mL)

CTAB (Hexadecyl-trimethyl amonium bromide) 2 g

1 M Tris.Cl (pH: 8.0) 10 mL

0.5 M EDTA (pH: 8.0) 4 mL

5 M NaCl 28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.

5X CTAB Extraction Solution (100 mL)

CTAB (Hexadecyl-trimethyl amonium bromide) 5 g

1 M Tris.Cl (pH: 8.0) 10 mL

0.5 M EDTA (pH: 8.0) 4 mL

5 M NaCl 28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.

APPENDIX H: Sequence Analysis Conditions and Results

CaMV 35S

Primers :

35S – 1 (sense) : 5' – GCT CCT ACA AAT GCC ATC AT T – 3'
35S – 2 (antisense) : 5' – CTC CAA ATG AAA TGA AC – 3'

Annealing T = 45ºC

Reaction mixture

Reagent	Volume
10 X Rxn Buffer	3 µl
25 mM $MgCl_2$	1.5 µl
2.5 mM dNTP	2 µl
10 µM Primer F	3 µl
10 µM Primer R	3µl
dH_2O	16 µl
Taq	0,5 µl
Template DNA	1 µl
Total volume	30 µl

PCR conditions

95ºC for 5 min (initial denaturation)
35 cycles of
94ºC for 1 min (denaturation)
45ºC for 1 min (annealing)
72ºC for 2 min (extension)
72ºC for 7 min (final extension)

NOS terminator

Primers :

T_{NOS} – 1 (sense) : 5'- GAA TCC TGT TGC CGG TCT TG- 3' T_m = 62ºC
T_{NOS} – 2 (antisense) : 5'- TTA TCC TAG TTT GCG CGC TA- 3' T_m = 58 ºC

Annealing T = 55ºC

Reaction mixture

Reagent	Volume
10 X Rxn Buffer	3 µl
25 mM $MgCl_2$	1.5 µl
2.5 mM dNTP	2 µl
10 µM Primer F	3 µl
10 µM Primer R	3µl
dH_2O	16 µl
Taq	0,5 µl
Template DNA	1 µl
Total volume	30 µl

PCR conditions

95°C for 7 min (initial denaturation)
35 cycles of
94°C for 30 sec (denaturation)
55°C for 45 sec (annealing)
72°C for 90 sec (extension)
72°C for 7 min (final extension)

Pflp

Primers :

AgSP5 (sense) : 5' – CGG GAT CCC GAT GGC TAG TGT CTC AGC TAC CA – 3' T_m = 68ºC
Ag3 (antisense) : 5' – CGA GCT CGT TAG CCC ACG AGT TCT GCT TCT – 3' T_m = 66 ºC

Annealing T = 62° C

Reaction mixture

Reagent	Volume
10 X Rxn Buffer	3 µl
25 mM $MgCl_2$	1.5 µl
2.5 mM dNTP	2 µl
10 µM Primer F	2 µl
10 µM Primer R	2 µl
dH_2O	18 µl
Taq	0,5 µl
Template DNA	1 µl
Total volume	30 µl

PCR conditions

95ºC for 4 min (initial denaturation)
35 cycles of
94°C for 1 min(denaturation)
55°C for 1 min (annealing)
72°C for 2 min (extension)
72°C for 7 min (final extension)

After PCR, gel electrophoresis has been carried out to estimate amplicon concentrations.

The Results of Sequence Analysis.

35S F

```
Binary vector pCAMBIA-1304, complete sequence, Length=12361

 Score =  289 bits (146),  Expect = 4e-75
 Identities = 174/183 (95%), Gaps = 1/183 (0%)
 Strand=Plus/Plus

Query  9     68
AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGA
Sbjct  12145  12204

Query  69     128
AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGGATGTGANTNNCCCTGGG
|||||||||||||||||||||||||||||||||||| |||||||     ||   |
AAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATT-GATGTGATATCTCCACTG
Sbjct  12205  12263
```

```
Query  129    188
ACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAA
Sbjct  12264  12323

Query  189    GTT  191
              |||
Sbjct  12324  GTT  12326
```

Score = 276 bits (139), Expect = 6e-71
Identities = 181/192 (94%), Gaps = 3/192 (1%)
Strand=Plus/Minus

```
Query  1      59
TATCGTTCA-GATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG
||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
TATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG
Sbjct  10771  10712

Query  60     119
CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGGATGTGANT
|||||||||||||||||||||||||||||||||||||||||||||||| |||||||
CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATT-GATGTGATA
Sbjct  10711  10653

Query  120    179
NNCCCTGGGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTA
   ||   |||||||||||||||||||||||||||||||||||||| ||||||||||
TCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGA-CCTTCCTCTA
Sbjct  10652  10594

Query  180    TATAAGGAAGTT  191
              ||||||||||||
Sbjct  10593  TATAAGGAAGTT  10582
```

Score = 204 bits (103), Expect = 2e-49
Identities = 110/111 (99%), Gaps = 1/111 (0%)
Strand=Plus/Minus

```
Query  1      59
TATCGTTCA-GATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG
||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
TATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG
Sbjct  11098  11039

Query  60     110
CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTG
|||||||||||||||||||||||||||||||||||||||||||||||||||
CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTG
Sbjct  11038  10988
```

35R

Binary vector pCAMBIA-1304, complete sequence, Length=12361

Score = 357 bits (180), Expect = 2e-95
Identities = 180/180 (100%), Gaps = 0/180 (0%)
Strand=Plus/Plus

```
Query  34     93
TTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGT
Sbjct  10624  10683

Query  94     153
GGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACC
Sbjct  10684  10743

Query  154    213
ACTGTCGGCAGAGGCATCTTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACTGTCGGCAGAGGCATCTTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTA
Sbjct  10744  10803
```

Score = 341 bits (172), Expect = 1e-90
Identities = 178/180 (98%), Gaps = 0/180 (0%)
Strand=Plus/Minus

```
Query  34     93
TTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGT
Sbjct  12283  12224

Query  94     153
GGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACC
Sbjct  12223  12164

Query  154    213
ACTGTCGGCAGAGGCATCTTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTA
|||||||||||||||||||| |||||| ||||||||||||||||||||||||||||||||
ACTGTCGGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATGGCATTTGTA
Sbjct  12163  12104
```

Score = 299 bits (151), Expect = 4e-78
Identities = 151/151 (100%), Gaps = 0/151 (0%)
Strand=Plus/Plus

```
Query  63     122
TATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGC
Sbjct  10980  11039

Query  123    182
TCCTCGTGGGTGGGGGTCCATCTTTGGGACCACTGTCGGCAGAGGCATCTTGAACGATAG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TCCTCGTGGGTGGGGGTCCATCTTTGGGACCACTGTCGGCAGAGGCATCTTGAACGATAG
Sbjct  11040  11099

Query  183    CCTTTCCTTTATCGCAATGATGGCATTTGTA  213
              ||||||||||||||||||||||||||||||
Sbjct  11100  CCTTTCCTTTATCGCAATGATGGCATTTGTA  11130
```

NOS F

Binary vector pCAMBIA-1305.1, complete sequence, Length=11846

Score = 143 bits (72), Expect = 6e-31
Identities = 76/78 (97%), Gaps = 0/78 (0%)
Strand=Plus/Plus

```
Query  39    98
ACATGTNATNCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTAT
|||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
ACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTAT
Sbjct  2189  2248

Query  99    ACATTTAATACGCGATAG  116
              ||||||||||||||||||
Sbjct  2249  ACATTTAATACGCGATAG  2266
```

NOS R

Binary vector pCAMBIA-1305.1, complete sequence, Length=11846

Score = 145 bits (73), Expect = 2e-31
Identities = 112/119 (94%), Gaps = 5/119 (4%)
Strand=Plus/Minus

```
Query  27    85
TGCGGGANTCTAATCATAAAA-CCCATCTCATAAATAACGTCATGGCATTACATGGTTAA
||||||| ||||||||||||| |||||||||||||||||||||| ||||||||| ||||
TGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATG-CATTACATG-TTAA
Sbjct  2243  2186

Query  86    144
TTATTACATGGCTTAACGTAATTCAACAGAAATTATATGATANTCATACGCAAGACCGG
|||||||||| |||||||||||||||||||||||||||| |||| ||||||||||||
TTATTACATG-CTTAACGTAATTCAACAGAAATTATATGATAATCAT-CGCAAGACCGG
Sbjct 2185 2129
```

***Pflp* Ag3**

Capsicum annuum ferredoxin-like protein (ap1) mRNA, complete cds
Length=662

Score = 765 bits (386), Expect = 0.0
Identities = 396/398 (99%), Gaps = 1/398 (0%)
Strand=Plus/Plus

```
Query  10   69
CTCCTTCATGCCAAGAAAACCAGCTGTGACAAGCCTTAAACCCATCCCAAACGTTGGGGA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTCCTTCATGCCAAGAAAACCAGCTGTGACAAGCCTTAAACCCATCCCAAACGTTGGGGA
Sbjct  92   151

Query  70   129
AGCACTGTTTGGGCTTAAATCAGCAAATGGTGGCAAAGTCACTTGCATGGCTTCATACAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGCACTGTTTGGGCTTAAATCAGCAAATGGTGGCAAAGTCACTTGCATGGCTTCATACAA
Sbjct  152  211

Query  130  189
AGTGAAACTTATCACACCTGACGGACCAATAGAATTTGATTGCCCAGATGATGTGTACAT
|||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
AGTGAAACTTATCACACCTGACGGACCAATAGAATTTGATTGCCCAGATAATGTGTACAT
Sbjct  212  271

Query  190  249
TCTTGATCAAGCTGAGGAAGCAGGACATGATCTTCCTTATTCGTGCAGGGCAGGTTCTTG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TCTTGATCAAGCTGAGGAAGCAGGACATGATCTTCCTTATTCGTGCAGGGCAGGTTCTTG
Sbjct  272  331

Query  250  309
CTCATCTTGTGCTGGTAAAATTGCTGGTGGAGCTGTTGATCAAACTGATGGCAACTTTCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTCATCTTGTGCTGGTAAAATTGCTGGTGGAGCTGTTGATCAAACTGATGGCAACTTTCT
Sbjct  332  391

Query  310  369
TGATGATGACCAATTAGAGGAGGGATGGGTGCTAACTTGTGTTGCTTATCCACAGTCTGA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGATGATGACCAATTAGAGGAGGGATGGGTGCTAACTTGTGTTGCTTATCCACAGTCTGA
Sbjct  392  451

Query  370  TGTTACTATTGAGACTCACAAAGA-GCAGAACTCGTGG  406
            |||||||||||||||||||||||| ||||||||||||
Sbjct  452  TGTTACTATTGAGACTCACAAAGAGGCAGAACTCGTGG  489
```

***Pflp* Ag5**

Capsicum annuum ferredoxin-like protein (ap1) mRNA, complete cds
Length=662

Score = 724 bits (365), Expect = 0.0
Identities = 368/369 (99%), Gaps = 0/369 (0%)
Strand=Plus/Minus

```
Query  34   93
ACACAAGTTAGCACCCATCCCTCCTCTAATTGGTCATCATCAAGAAAGTTGCCATCAGTT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACACAAGTTAGCACCCATCCCTCCTCTAATTGGTCATCATCAAGAAAGTTGCCATCAGTT
Sbjct  433  374

Query  94   153
TGATCAACAGCTCCACCAGCAATTTTACCAGCACAAGATGAGCAAGAACCTGCCCTGCAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGATCAACAGCTCCACCAGCAATTTTACCAGCACAAGATGAGCAAGAACCTGCCCTGCAC
Sbjct  373  314

Query  154  213
GAATAAGGAAGATCATGTCCTGCTTCCTCAGCTTGATCAAGAATGTACACATCATCTGGG
|||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
GAATAAGGAAGATCATGTCCTGCTTCCTCAGCTTGATCAAGAATGTACACATTATCTGGG
Sbjct  313  254

Query  214  273
CAATCAAATTCTATTGGTCCGTCAGGTGTGATAAGTTTCACTTTGTATGAAGCCATGCAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAATCAAATTCTATTGGTCCGTCAGGTGTGATAAGTTTCACTTTGTATGAAGCCATGCAA
Sbjct  253  194

Query  274  333
GTGACTTTGCCACCATTTGCTGATTTAAGCCCAAACAGTGCTTCCCCAACGTTTGGGATG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GTGACTTTGCCACCATTTGCTGATTTAAGCCCAAACAGTGCTTCCCCAACGTTTGGGATG
Sbjct  193  134

Query  334  393
GGTTTAAGGCTTGTCACAGCTGGTTTTCTTGGCATGAAGGAGGTACTAATCATGGTAGCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGTTTAAGGCTTGTCACAGCTGGTTTTCTTGGCATGAAGGAGGTACTAATCATGGTAGCT
Sbjct  133  74

Query  394  GAGACACTA  402
             |||||||||
Sbjct  73   GAGACACTA  65
```

APPENDIX I: TRI reagent and Northern Blot Solutions

TRI Reagent (110 mL)

Guanidine thiocyanate 23.7 g
100 mM Na-citrate pH: 7.0 12.5 mL
35% Sarcosyl 715 µL
β-mercaptoethanol 340 µL
Water saturated phenol 50 mL
2 M Potassium acetate pH: 4.8 10 mL

Guanidine thiocyanate and 100 mM Na-citrate pH: 7.0 are dissolved in DEPC-treated distilled water and the volume was completed to 45 mL. Then 35% sarcosyl and β-mercaptoethanol was added and the volume was completed to 50 mL. The solution was autoclaved. 50 mL water saturated phenol was added onto the solution in hood and 10 mL sterile 2M potassium acetate pH: 4.8 was added.

10X MOPS (500 mL)

2.05 g Sodium acetate
1.46 g EDTA
20.92 g MOPS (3-N-[Morpholino] propane sulfonic acid)
All components were mixed and the volume was completed to 500 mL. PH was adjusted to 7 and the solution was filter sterilized.

Sample Buffer (for 500 µL, prepare freshly)

250 µL 100% formamide
85.75 µL 37% formaldehyde
50 µL 10X MOPS
2.5 µL ethidium bromide
All components were mixed the volume completed to 500 µL.

Formaldehyde Gel (for 150 mL)

1.5 g agarose

15 mL 10X MOPS

25.7 mL 37% formaldehyde

Agarose and MOPS were dissolved in DEPC-treated distilled water with microwave oven. 2 μL of ethidium bromide were added and the gel was cooled. Then formaldehyde was added under the hood and the gel was poured. The gel was run with 1X MOPS as buffer.

Transfer Buffer (20X SSC)

88.23 g Tri-sodium citrate

175.32 g NaCl

All are dissolved in 800 mL dH_2O and pH is adjusted to 7-8 then the volume is completed to 1 L with distilled water.

Pre-hybridization and Hybridization Buffer

NaCl 0.25 mL/cm^2 hybridization buffer

Blocking regent 4%

NaCl was added into hybridization buffer and the solution was stirred at room temperature. Then blocking reagent was added and the solution was mixed for 1-2 hours at room temperature until completely dissolved.

Primary Wash Buffer (1L)

		Final concentration
Urea	120 g	2 M
SDS	1 g	0.1%
0.5 M sodium phosphate (pH: 7.0)	100 mL	50 mM
Sodium chloride	8.7 g	150 mM
1 M magnesium chloride	1 mL	1 mM
Blocking reagent	2 g	0.2%

All the components were added and the volume was completed to 1 L with distilled water. Primary wash buffer can be kept at 2-8 ^{0}C up to one week.

Secondary Wash Buffer (for 500 mL)

Tris-base	60.5 g
Sodium chloride	56 g

All components were dissolved in water and the volume is completed to 500 mL.

Secondary Wash Buffer Working Solution

Secondary wash buffer was diluted 1:20 and 2 mL/L of 1M magnesium chloride was added (2 mM magnesium in solution).

Secondary wash buffer can not be stored, it should be used immediately